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Ipratropium Bromide Mediated Myocardial Injury in in vitro Models of Myocardial Ischaemia/Reperfusion

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By

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Abstract

Ipratropium bromide is a short-acting, non-selective, muscarinic antagonist frequently prescribed for the treatment of Chronic Obstructive Pulmonary Disease (COPD) and as an emergency adjunct therapy for acute asthma. Within the past decade, there has been an accumulating wealth of clinical evidence which indicates that anti-muscarinic drugs, such as ipratropium, are responsible for an increased risk of stroke or, an adverse cardiovascular outcome (including increasing the risk and severity of myocardial infarction (MI)).

MI remains the highest risk factor of death for COPD patients due to the systemic co-morbidities associated with COPD, which includes ischaemic heart disease (IHD). Despite the knowledge that approximately 22% of COPD patients also suffer from underlying IHD, the cardiovascular safety of muscarinic antagonists, such as ipratropium, has not been tested in a non-clinical setting of IHD or MI. In order to address this, the current project was designed to investigate, for the first time, the effects of ipratropium on the myocardium in a non-clinical setting.

It was identified that under normoxic conditions, ipratropium did not have a significant effect on cardiac myocyte viability or infarction, from 3 month Sprague Dawley rats. In addition to this, following simulated ischaemia, ipratropium also did not appear to exacerbate myocardial injury. However, when ipratropium was administered in the context of simulated ischaemia followed by reperfusion, there was a significant exacerbation in myocardial injury which was characterised by increases in infarction, apoptosis, necrosis and a loss of resilience of oxidative stress.

In order to characterise the mechanism by which ipratropium exerts the observed cardio-toxic effects, it was investigated whether acetylcholine (ACh) or cyclosporin A (CsA) were capable of attenuating the ipratropium induced cardiotoxicity. Both agents showed significant limitation of injury when co-administered with ipratropium indicating that ipratropium exerts its cardio-toxic effect through a mechanism which links muscarinic signalling to the mitochondrial permeability transition pore (mPTP). This supports previously published work where the protective signalling of ACh has been shown to promote the phosphorylation of pro-survival kinases, such as Akt and Erk1/2 and that this provides inhibition of the mPTP.

Western blotting was employed to identify whether there was an involvement of the pro-survival kinases Akt and Erk1/2, as well as the stress induced kinase JNK. Ipratropium significantly increased levels of phospho-Akt and phospho-Erk1/2. However, JNK levels appeared to be insignificantly altered in comparison with the control groups. Both ACh and CsA were capable of limiting these increases.

Further to this, an aged study was carried out, which showed that, within the aged myocardium, ipratropium is capable of eliciting further injury in comparison with the 3 month age groups. The effect of ipratropium on tolerance of oxidative stress was not significant, but, also, ACh and CsA were shown as unable to protect. Significant levels of JNK were also observed in the aged animals in comparison with the 3 month groups.

In combination, the results presented here demonstrate, for the first time, that ipratropium is capable of exacerbating ischaemia/reperfusion injury in *in vitro* models of myocardial ischaemia/reperfusion. In addition, ACh and CsA are capable of limiting this injury, implying a role for pro-survival kinases and the mPTP in ipratropium

induced myocardial injury. In the aged study, ipratropium still exacerbated injury, however, ACh and CsA appeared unable to protect, therefore promoting previous work that cellular signalling is altered in the senescent myocardium. In conclusion, further studies must be carried out in order to fully characterise the cardio-vascular safety profile of ipratropium.

Publications from this Thesis

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List of Abbreviations

ACh	Acetylcholine
AIF	Apoptosis inducing factor
AKT	Cellular AKT/protein kinase B
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocator
Apaf-1	Apoptosis protease activating factor-1
ATP	Adenosine Tri-phosphate
Atr	Atropine
BAD	Bcl-2 family, BH-3 only domain, pro-apoptotic protein
BAK protein	Bcl-2 family, BH-1, BH-2 & BH-3 domain, pro-apoptotic
BAX protein	Bcl-2 family, BH-1, BH-2 & BH-3 domain, pro-apoptotic
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
Caspase	Cysteine aspartate specific protease
CF	Coronary flow
CBN	Contraction band necrosis
CHD	Coronary heart disease
CsA	Cyclosporin A
Cyt-C	Cytochrome C
DEVD	Z-DEVD-FMK, Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)--ValAsp(OMe)-fluoromethylketone, cell permeable, irreversible caspase-3 inhibitor

DIABLO/SMAC	Direct IAP-binding protein with low pI/second mitochondria-derived activator of caspases
DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
ERK	Extracellular signal regulated kinase
FADD	Fas associated death domain
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
H/R	Hypoxia/Re-oxygenation
HR	Heart rate
I/R	Ischaemia/Reperfusion
I/R%	Infarct size to Risk ratio
IHD	Ischaemic heart disease
Ip	Ipratropium bromide
JNK	c-jun N-terminal kinase
KHB	Krebs-Heinsleit buffer
LVDP	Left ventricular developed pressure
MAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
mPTP	Mitochondrial permeability transition pore
MOMP	Mitochondrial Outer Membrane Permeabilisation
MTT bromide	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NO	Nitric oxide
PKC	Protein Kinase C
PMNs	Polymorphonuclear leucocytes
RISK	Reperfusion injury salvage kinase
SEM	Standard error of the arithmetic mean

Tio	Tiotropium bromide
TMRM	Tetramethylrhodamine, Methyl Ester
TTC	2,3,5-triphenyl-2H-tetrazolium-chloride
VDAC	Voltage dependent anion channel
Wort	Wortmannin

Chapter 1 Literature review

1.0 Introduction

The clinical manifestation of ischaemia/reperfusion is more commonly known as acute myocardial infarction (MI). Within the UK, approximately 80,000 individuals suffer from MI annually, of which many are predisposed to the condition due to underlying, diagnosed or un-diagnosed, ischaemic heart disease (IHD) (Dorsch et al. 2008). Chronic obstructive pulmonary disease (COPD) represents a significant, and increasing cause of morbidity and mortality across the globe, accounting for the fourth highest cause of deaths, globally, every year (Singh, Loke and Furberg 2008). In addition to this, the pathology of COPD is associated with approximately a 52% increased risk in mortality due to cardiovascular events (Singh et al. 2011), with many COPD patients also suffering from underlying IHD. It is estimated that around 22% of COPD patients will die from complications following a myocardial ischaemic event and, in particular, from MI (Lofdahl et al. 2007).

In 2008, a meta-analysis of COPD patients was released by Singh *et al.* which indicated that the administration of anti-cholinergic (muscarinic acetylcholine receptor antagonist) drugs to COPD patients increased the risk and severity of a stroke or cardiovascular event (Singh, Loke and Furberg 2008). Anti-cholinergic compounds are the most commonly prescribed treatment for COPD, due to their ability to antagonise the M_3 muscarinic receptors within the respiratory system, thereby alleviating bronchial spasm, symptomatic of COPD exacerbations, facilitating bronchodilation, due to their bronchodilatory effect (the specific mechanism of action is discussed in section

1.9.4). However, despite the known co-morbidities between COPD and IHD, there have been no pre-clinical investigations to assess the effects of anti-cholinergic compounds, such as ipratropium bromide (a non-selective muscarinic receptor antagonist), in the setting of IHD or MI.

1.1 Chronic Obstructive Pulmonary Disease (COPD)

COPD, as defined by the Global initiative for Chronic Obstructive Lung Disease (GOLD), is a lung pathology associated with airflow obstruction that is not fully reversible (Singh et al. 2008). According to the World Health Organisation, COPD affects approximately 64 million people and constitutes the most common chronic respiratory disease globally (Berndt, Leme and Shapiro 2012). By 2020, it is estimated that COPD will be responsible for the 3rd highest number of deaths due to disease, annually, throughout the world (Mannino and Kiriz 2006). COPD is an umbrella term which includes emphysema and chronic bronchitis and accounts for around 5% of all deaths within the UK annually (Feary et al. 2010). The primary diagnostic criterion is a spirometry result showing an irreversible, decreased, ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC ratio) (Berndt, Leme and Shapiro 2012). The major pathophysiology of COPD is chronic inflammation of the respiratory system, including the bronchi, lung tissue and pulmonary vasculature (Asano et al. 2010). The primary causes of COPD are due to environmental irritants, which may be occupational but the major cause is tobacco smoke (Baur, Bakehe and Vellguth 2012).

Despite this, the involvement of genetic factors also plays a fundamental role in determining COPD development (Almansa et al. 2012). As with many complex disease states the pathogenic mechanisms of COPD, due to genetic predispositions, are challenging to unravel. This is, partially, due to the associated systemic pathologies which accompany COPD as well as the heterogeneous nature of the disease (Nakamura 2011). For example, the irreversible airflow obstruction necessary for a diagnosis of COPD may result from the loss of elastic recoil of the alveoli, symptomatic of emphysema, or due to obstruction throughout the bronchi and bronchioles (bronchitis) (Almansa et al. 2012). Alternatively, COPD may be a consequence of both pathologies (Berndt, Leme and Shapiro 2012). Despite both disorders occurring as a consequence of cigarette smoking they are not actually mechanistically related, therefore the elucidation of genetic determinants for either pathology is not necessarily sufficient to determine the concurrence of both in COPD patients (Berndt, Leme and Shapiro 2012).

Despite these difficulties, however, COPD development has been strongly linked to a genetic disorder known as alpha-1 antitrypsin (AAT) deficiency (Foreman, Campos and Celedon 2012). AAT is encoded by the SERPINA1 gene and primarily synthesised in the liver (Thun et al. 2013). The primary role of ATT is as an endogenous inhibitor of neutrophil elastase (a serine protease secreted by neutrophils and macrophages which, during inflammatory immune responses, destroy both bacteria and host tissue) (Stoller and Aboussouan 2012).

In the context of COPD, AAT deficiency was first identified in 1964 where two patients with severe emphysema, which progressed to loss of lung compliancy due to the

subsequent development of chronic bronchitis, were reported (Eriksson 1964). The loss of AAT exacerbates COPD development (even in patients who have never smoked) by mimicking the features of smoking-related COPD via production of alveolar lesions, characteristic of emphysema (Stockley 1999). Interestingly, in COPD patients without AAT deficiency, disease development has also been linked to an imbalance between anti-protease activity and increased transcriptional activity of neutrophil protease genes (Stockley 1999, Almansa et al. 2012). This indicates mechanistic similarity of COPD development despite whether the initial impetus is due to environmental or genetic factors.

Irrespective of the cause, COPD causes partially reversible airflow obstruction, which is characterised by shortness of breath, coughing and wheezing. The primary cellular response is an inflammatory immune response comprising of neutrophils, $CD8^+$ cytotoxic T-lymphocytes and macrophages (Almansa et al. 2012). Despite the anatomical differences of bronchitis and emphysema, both pathologies predominantly result from a destructive, neutrophil driven, immune response (Barnes and Celli 2009). Following inflammatory stimuli, such as cigarette smoke and environmental pollutants, neutrophils are recruited to the lungs, primarily via the actions of alveolar macrophages (Stockley 1999). This permits the release of pro-inflammatory cytokines, for example Il-1 and TNF-α, leading to a downstream immunologic inflammatory signalling cascade and hyper-secretion of mucus (Barnes and Celli 2009). The excess mucus secretion exacerbates the inflammatory response and promotes bronchitis due to an associated accumulation of bacteria (Figure 1.1).

Figure 1.1: Schematic to show the inflammatory immune cells and mechanisms involved in COPD development, including the mucus hyper-secretion symptomatic of chronic bronchitis and alveolar wall destruction as is typical of emphysema (adapted from Barnes and Celli 2009).

In addition to airflow obstruction, there is increasing evidence that COPD is also responsible for wider systemic pathologies, including diabetes mellitus, stroke and cardiovascular disease (Macnee, Maclay and McAllister 2008). This indicates that the morbidity and mortality rate from death associated with COPD may be much higher than death directly due to COPD. In particular, IHD is a well known co-morbidity associated with COPD (Huiart, Ernst and Suissa 2005, Maclay, McAllister and Macnee

2007, Macnee, Maclay and McAllister 2008). Underlying IHD also represents the highest risk factor for death by myocardial infarction (MI) in COPD sufferers (Zielinski et al. 1997). It is estimated that approximately 22% of patients with COPD suffer from underlying IHD (Lofdahl et al. 2007). Currently, the association between COPD and IHD is attributed to underlying systemic inflammation associated with both pathologies (Barnes and Celli 2009). However, despite the recent association with anti-cholinergic treatments and CVD, patients with COPD and underlying IHD have not been tested in pre-clinical trials for bronchodilator drugs, including anti-cholinergics such as ipratropium.

As presented in table 1.1, whether anti-cholinergic treatment is associated with an increase in adverse cardiovascular outcomes remains as a matter of some contention. There is approximately an equal split between studies which show a positive association versus those which show a negative association of anti-cholinergics with regards to adverse cardiovascular outcomes (Hilleman et al. 2009). The potential difference in conclusions from these studies could be explained by a difference in the end points which were investigated. Also, for some studies, underlying heart conditions, such as CVD, MI or IHD, were not assessed at all (Singh, Loke and Furberg 2008).

Table 1.1: A comprehensive review of studies which aimed to identify whether inhaled anti-cholinergics were associated with risks of adverse cardiovascular outcomes in patients with COPD (taken from (Hilleman et al. 2009).

1.2 Ischaemic heart disease (IHD)

Globally there are approximately 16.7 million deaths due to cardiovascular disease (CVD) every year, of which around 7.2 million deaths are attributed to ischaemic heart disease (IHD) (Mendis et al. 2004). With respect to COPD patients, IHD is a common co-morbidity, with approximately 22% of COPD patients also have a diagnosis of IHD, however, in reality, it is likely that many COPD patients, with underlying IHD, have not received a diagnosis for both pathologies (Barnes and Celli 2009).

IHD is characterised by myocardial ischaemia, whereby blood supply to myocardial tissue is reduced. This is most commonly due to the build up of atherosclerotic plaques in the coronary arteries (Ross 1999). This leads to occlusion of the coronary arteries, although even a 50% reduction in the diameter of the coronary arteries is sufficient for an ischaemic event to occur (Hoffman et al. 2004). Within the UK, IHD is responsible for approximately 82,000 deaths per year and affects approximately one fifth and one eighth of men and women, respectively. As such, IHD constitutes both the leading cause of death globally and within the UK (Brayton et al. 2012). In addition, IHD also has been established as the leading cause of global burden as well as substantially reducing quality of life, as well as life expectation, for patients (Allender et al. 2007).

The incidence and risk of IHD is primarily associated with increased age accompanied by hypercholesterolemia and hypertension (Brayton et al. 2012). Especially within Western society, smoking, inactivity, diabetes and diet have been shown to majorly influence the development of atherosclerosis and subsequent IHD (Baur, Bakehe and Vellguth 2012).

From a clinical perspective, IHD frequently manifests in a multitude of symptoms such as arrhythmias, angina, heart failure and myocardial infarction (MI), although frequently patients do not present with symptoms until after they have experienced a severe ischaemic insult or MI (Misra et al. 2009). MI remains the most serious and often fatal consequence of IHD (Lofdahl et al. 2007), with myocardial infarct size as a determining factor for long term prognosis (Ferreira 2010). The primary therapeutic option is myocardial revascularisation (a method which restores blood flow to the myocardium) in order to salvage myocardial tissue, thereby limiting infarct size and optimise clinical outcome (Lee et al. 2008). Currently, thrombolytic therapy, for example streptokinase, first administered in 1974 by Braunwald *et al.* (Ferreira 2010), and primary percutaneous coronary artery angioplasty are the most commonly used interventions (Hausenloy, Tsang and Yellon 2005).

1.3 Pathogenesis of myocardial infarction

Myocardial infarction develops as a clinical manifestation of IHD and frequently presents as myocardial ischaemia, whereby blood, and thus oxygen, flow is limited to areas of the myocardium with resultant cardiac myocyte loss due to cessation of oxidative phosphorylation (Hausenloy and Yellon 2013). Paradoxically, upon restoration of blood flow to the ischaemic tissue (reperfusion), further, irreversible damage is caused (Hausenloy et al. 2005). The exact mechanism by which ischaemia/reperfusion (I/R) injury occurs as a consequence of diverse cellular signalling cascades remains elusive, however it is known that myocyte death occurs via both

apoptosis and necrosis (Kung, Konstantinidis and Kitsis 2011), with a fundamental role for the mitochondrial permeability transition pore (mPTP). Figure 1.2 represents a simple overview of the major pathways which lead to myocyte loss following I/R (Brookes and Darley-Usmar 2004).

In the neonatal heart, cardiac myocytes become terminally differentiated, and therefore lose the ability to proliferate (Kuwahara et al. 2000). It is therefore essential that functional myocytes are salvaged following I/R in order to ensure the myocardium can adequately function following this injury (Hausenloy and Yellon 2004).

Figure 1.2: Schematic to represent the basic mechanisms leading to myocyte loss following I/R. I/R induces increases in Ca^{2+} and reactive oxygen species (ROS), which therefore permits opening of the mPTP. Primary necrosis occurs as a result of depleted ATP levels. The release of mitochondrial pro-apoptotic proteins into the cytosol also initiates apoptotic mechanisms. However, these pathways are interlinked as, if ATP levels are too low, apoptosis is unable to occur, and thus myocyte fate will be determined by necrosis, rather than apoptosis (Adapted from (Brookes and Darley-Usmar 2004)).

1.3.1 Ischaemia

Despite having been one of the most extensively studied topics in cardiovascular research, the exact definition of myocardial ischaemia remains as a matter of some debate (Hearse 1994). Rudolf Virchow first proposed the term “ischaemia” in 1858, which translates as: “... I propose the new term ischaemia in order to describe the hindrance of blood supply and the increase in the resistance of influx” (Letterer 1958). Throughout this work, the definition is determined as the conditions by which the oxygen and nutrient supply to the myocardium are insufficient to meet myocardial demand (Asano et al. 2010). Physiologically, myocardial ischaemia occurs as a consequence of a reduction, or entire cessation, of blood flow to the heart. This causes a reduction in oxidative phosphorylation (Figure 1.3) and, consequently, reduced ATP formation (Murphy and Steenbergen 2008). Cardiac myocytes, the contractile cells within the myocardium, rely heavily on adequate ATP supply, produced via oxidative phosphorylation, primarily in the electron transport chain, within the mitochondria (Hausenloy and Yellon 2004). As a result, following ischaemia, myocytes resort to anaerobic respiration (the conversion of glucose to lactic acid) to meet the energy demands of the heart. However, this process leads to an accumulation of lactic acid within the ischaemic region of the heart (Solaini and Harris 2005).

Figure 1.3: Schematic to represent the electron transport chain (ETC) which occurs on the mitochondrial membrane. Per each ETC cycle, ten $NADH^{+}$, a consequence of glycolysis, the conversion of pyruvate to acetyl-coenzyme A and from the citric acid cycle, enter the ETC. Two molecules of $FADH_2$ are also required and originate from the citric acid cycle. The ETC is comprised of five protein complexes, the first two of which systematically oxidise NADH (complex I) and $FADH_2$ (complex II), thereby providing electrons for the chain. At complex IV, the electrons are transferred to oxygen molecules, therefore reducing the oxygen to H_2O. Concomitantly, protons are pumped out of the mitochondrial intermembrane space and into the cytosol, therefore causing a net negative charge in the mitochondrial matrix and net positive charge in the intermembrane space. It is this electrochemical gradient which then permits the synthesis of ATP in complex V (adapted from Chaban, Boekema and Dudkina 2014).

The severity and length of ischaemia are primarily the factors on which myocyte loss are dependent (DeBoer et al. 1983). For example, an ischaemic event of less than 20 minutes results in reversible myocardial injury whereby, despite initial depression of contractile function, the myocardium may eventually completely recover (Davis et al. 2012). Following an ischaemic attack of 15 minutes, ATP levels are depleted by approximately 65%. This is further increased to a reduction of 90% if ischaemia occurs for 40 minutes (Reimer and Jennings 1986). The ability of the myocardium to maintain ion homeostasis and generate sufficient force is therefore lost, leading to myocyte necrosis, characterised by acidosis, organelle disorganisation and swelling of the

myocytes prior to subsequent cellular death (Maxwell and Lip 1997). Necrotic cardiac myocyte death due to ischaemia is referred to as myocardial infarction (Braunwald and Kloner 1985).

The inability of cardiac myocytes to meet ATP demand also causes cessation of function of ATP dependent Ca^{2+} and Na^{+}/K^{+} ion pumps and ion exchangers and transporters (Schafer et al. 2001). This promotes K^{+} accumulation in the extracellular matrix and an inability for cardiac myocytes to maintain healthy, physiological intracellular concentrations of Na^{+} and Ca^{2+} (Wang et al. 2012). The majority of Ca^{2+} removal from the intracellular space is via the Na^{+}/Ca^{2+} exchanger (NCX), with a very minor contribution from SERCA (Sarcoplasmic reticulum/Endoplasmic Reticulum Ca^{2+} ATPase) (Murphy and Steenbergen 2008). The NCX is an antiporter membrane protein responsible for the removal of intracellular Ca^{2+} (Lee, Dhalla and Hryshko 2005). For over twenty-five years, it has been indicated that a rise in cytosolic Ca^{2+} precedes irreversible myocardial injury following an ischaemic insult (Murphy and Steenbergen 2008). Under conditions of ischaemia, the NCX essentially functions in reverse, such that it serves to increase intracellular Ca^{2+} as opposed to maintaining Ca^{2+} homeostasis (Inserte et al. 2009). Although the increase in Ca^{2+} has been shown to precede irreversible myocyte injury rather than generate injury alone, the ability to attenuate Ca^{2+} homeostatic de-regulation is sufficient to delay the onset of irreversible myocardial injury (Murphy and Steenbergen 2008).

During ischaemia, ATP is degraded to adenosine monophosphate (AMP), which, consequently is further broken down thereby leading to the formation of free radicals (Glantzounis et al. 2005) (Figure 1.4).

Figure 1.4: Schematic representation of ROS formation following I/R. The mechanism involved shows that degradation of ATP to AMP subsequently leads to the formation of superoxide free radicals ($O_2^{\cdot-}$) which occurs during reperfusion/re-oxygenation. Xanthine oxidase produces uric acid from hypoxanthine and xanthine. This process is further involved in the formation of ROS via production of hydroxyl radicals (adapted from Glantzounis et al. 2005).

A plethora of work has been conducted to ascertain the role of reactive oxygen species (ROS) and their role in infarct development following myocardial ischaemia (Bolli et al. 1989) (described more fully in section 1.3.5). It has since been elucidated that, although following ischaemia, myocardial oxygen levels within the ischaemic zone do not immediately fall to zero and so, initially at least, oxygen is available to generate ROS (Davis et al. 2012). The quantities of ROS produced during ischaemia alone, are not sufficient to cause injury but contribute to the pathological outcome of I/R injury (Murphy and Steenbergen 2008).

1.3.2 Reperfusion injury

In order to prevent further irreversible, and potentially fatal, myocardial injury, it is necessary to reperfuse the myocardium following ischaemia. This is in order to salvage reversibly damaged myocytes, which is critical in order to maintain the integrity of as much of the myocardium as possible. This was first proposed by Herrick in 1912: "The hope for the damaged myocardium lies in the direction of securing a supply of blood through friendly neighbouring vessels so as to restore as far as possible its functional integrity" (Muller 1977). However, it was not until 1971 that Maroko et al proposed experimental studies designed to investigate the factors associated with infarct size (Maroko et al. 1972). Conversely, it has since been demonstrated that reperfusion itself is capable of eliciting further, irreversible, damage to the myocardium (Yellon and Hausenloy 2007b). The potentially detrimental aspect of myocardial reperfusion injury, termed "lethal reperfusion injury," has been defined as: as "myocardial injury caused by the restoration of coronary blood flow after an ischaemic episode (Yellon and Hausenloy 2007b). Therefore, the process of reperfusion causes further damage to the heart by inducing contractile dysfunction, microvascular impairment, lethal arrhythmias and also further myocyte death (Xiong et al. 2009, Hearse 2001). There have since been considerable efforts in the field to elucidate both the cellular mechanisms by which reperfusion injury is caused as well as measures with the potential to limit damage after a myocardial ischaemic insult.

The key determinant for the initiation of intrinsic apoptosis during reperfusion is mitochondrial outer membrane permeabilisation (MOMP), thereby initiating a caspase-dependent apoptotic cascade (Dewson and Kluck 2009). Increases of

intracellular Ca^{2+} and oxidative stress lead to rapid ATP depletion, thereby causing rupture of the outer mitochondrial membrane (Halestrap 2010). This facilitates the influx of toxic mitochondrial proteins into the cytosol, thus initiating apoptosis (Halestrap 2009, Halestrap and Pasdois 2009, Hausenloy et al. 2002). Myocyte death by necrosis also occurs (Yellon and Hausenloy 2007), via the resultant collapse of mitochondrial function after disturbance of mitochondrial membrane potential due to sustained increases in Ca^{2+} and the absence of adenine nucleotides (Crompton 1999). Ultimately, this leads to opening of the mitochondrial permeability transition pore (mPTP, described in further detail in section 1.7), which promotes primary necrosis due to depletion of ATP as well as apoptosis following outer mitochondrial membrane rupture (Miura and Tanno 2011).

The re-introduction of oxygen to the ischaemic myocardium has been consistently shown to cause a rapid increase in ROS (Murphy and Steenbergen 2008) (Figure 1.4). During reperfusion, in particular, this increase is associated with superoxide formation which then elicits myocyte damage via extensive oxidative stress (Pasdois et al. 2011). This is evidenced by the ability of antioxidants to reduce ROS levels and myocardial stunning (as described in section 1.3.3) (Buja and Weerasinghe 2010), however, whether the reduction in ROS can promote salvage of tissue, thereby reducing infarct size, remains as a matter of some controversy (Murphy and Steenbergen 2008). This is primarily due to the difficulties in translating experimental, animal models into clinical practice in humans as the contribution of ambient oxygen to ROS burst (as observed experimentally) is likely to be different from the oxygen levels inside the heart following ischaemia (Cascio et al 1992). As such, the pathological significance of ROS

and, hence potential benefits of reducing levels of ROS, in humans is still uncertain (Murphy and Steenbergen 2008). However, ROS scavengers have been shown as effective in the reduction of infarct size (Hayashida et al. 2008).

1.3.3 Stunned myocardium and hibernating myocardium

In the 1980s, two additional pathophysiological states due to I/R emerged, showing discrepancy between post-ischaemic myocardial contractile dysfunction and the other symptoms of ischaemia (Ferreira 2010). These were termed the stunned myocardium and hibernating myocardium, respectively. The principle is that, following reperfusion, there are regions of the myocardium which consist of viable myocytes, but with impaired function (Braunwald and Kloner 1985, Redwood, Ferrari and Marber 1998). First suggested by Heyndrickx et al in 1975, myocardial stunning is thought to occur due to the generation of oxygen free radicals or Ca^{2+} overload, both of which cause disruption of contractile proteins, thereby limiting contractility of the heart (Bolli et al. 1989, Ferreira 2010).

However, the hibernating myocardium is different from this as it relates to persistent left ventricular dysfunction (Bolli 1990). This was first described by Diamond, in 1978, "…ischaemic non-infarcted myocardium can exist in a state of functional hibernation" (Rahimtoola 1985). Both stunned and hibernating myocardium are characterised by dysfunctional, but still viable, cardiac myocytes, in contrast to infarction where myocytes are no longer viable (Bolli 1990). However, in a clinical context, contractile

dysfunction, as shown by ECG changes, may be sufficient injury to cause death, even in the absence of extensive infarction, or myocyte loss (Buja and Weerasinghe 2010).

1.3.4 No-reflow phenomenon

The no-reflow phenomenon occurs when blood flow to the ischaemic tissue is still impeded to regions of the myocardium despite relief of the cause of ischaemia (Rezkalla and Kloner 2002). Reperfusion is frequently initiated via the dissolution of an atherosclerotic plaque or the removal of the coronary artery occlusion (Kloner 2011). However, microvascular obstruction sometimes means that there is an inability for blood supply to be returned to areas of the ischaemic myocardium (Braunwald and Kloner 1985). It has been observed that endothelial swelling results in membrane bound blebs within the lumen which can plug the lumen, thereby hindering reperfusion (Kloner 2011).

1.3.5 Oxygen derived free radicals

Free radicals or reactive oxygen species (ROS) are a consequence of healthy, physiological processes, such as oxidative phosphorylation, and in the context of a healthy cell are usually generated by the mitochondria (Munday 2001). This free radical production is balanced by endogenous cellular anti-oxidant mechanisms, such as ROS catalysis by free radical scavengers, for example superoxide dismutase (Tsutsui, Kinugawa and Matsushima 2011). This is necessary, as a property of free radicals is the

presence of an unpaired electron, therefore enabling oxidative damage of other cellular components (Gustafsson and Gottlieb 2008). In particular, alterations and peroxidation of the proteins and phospholipids which comprise cell membranes, due to increases in free radical formation, leads to loss of membrane integrity and subsequent cell death (Dominguez-Rodriguez, Abreu-Gonzalez and Reiter 2014).

However, in the context of the ischaemic myocardium, anti-oxidant enzymes are insufficient to limit free radical induced injury as reperfusion with oxygenated blood causes an influx of oxygen derived free radicals to the ischaemic myocardium (Seddon, Shah and Casadei 2007). Upon introduction to the ischaemic myocardium, oxygen is rapidly reduced which leads to high levels of free radicals in the ischaemic myocardium within the first few minutes of reperfusion (Figure 1.4, Zweier 1988). The concept of oxygen derived free radicals as contributing to reperfusion injury was originally proposed by Hearse in 1973 (Hearse, Humphrey and Chain 1973) and, subsequently in 1987, free radical levels were shown to peak during reperfusion (Zweier, Flaherty and Weisfeldt 1987). Further to this, the activation and accumulation of neutrophils within the reperfused myocardium further stimulates free radical formation following I/R (Glantzounis et al. 2005, Jordan, Zhao and Vinten-Johansen 1999). In conjunction with this, ROS levels following muscarinic agonism by CDP-choline have been shown to be significantly reduced, with an associated reduction in apoptosis, following oxidative stress in cardiac myocytes (Gonzáles-Pacheco et al. 2014).

The production of ROS has also been shown to reduce endogenous levels of nitric oxide (Brown and Borutaite 2007). This may exacerbate endothelial injury and

influence the severity of microvascular dysfunction, contributing to contractile dysfunction as observed during myocardial stunning (Chen and Zweier 2014).

1.3.6 Polymorphonuclear leukocytes (PMNs)

Following I/R, an inflammatory immune response is elicited whereby chemotaxis recruits polymorphonuclear leukocytes (PMNs) to the ischaemic myocardium (Mehta, Nichols and Mehta 1988, Zhao et al. 2003). PMNs were initially associated with the inflammatory response observed following myocardial iscahemia/reperfusion injury in 1939 (Kenneth Mallory, White and Salcedo-Salgar 1939). It was presumed that the influx of PMNs to the ischaemic region of the myocardium represented an endogenous healing mechanism, activated as a pathophysiological response (Stangl et al. 2002). This notion was clinically demonstrated in the 1970s where the administration of the anti-inflammatory prednisone, in patients with acute myocardial infarction, resulted in ventricular aneurysm and rupture (Roberts, Kelliher and Lathers 1976). However, further to this, histological studies were conducted which showed a direct association between infarct development and the length of an ischaemic insult and the levels of PMN accumulation (Romson et al. 1983). This is primarily due to PMNs contributing as a major source of oxygen derived free radicals (Metha, Advani and Nadkarni 1988), therefore promoting oxidative stress and myocardial damage (Kaminski et al. 2002).

In addition to this, de-granulation of PMNs following immunogenic activation leads to release of potent enzymes which serve to hydrolyse and systematically breakdown the extracellular matrix. This has the consequence that myocardial tissue integrity cannot

be maintained with resultant myocyte death due to anoikis following loss of anchorage of myocytes from the extracellular matrix (Ikeda et al. 1998).

Despite controversy in this field, the majority of accumulating evidence supports that PMN accumulation in the ischaemic myocardium promotes injury. Most recently, pre-conditioning has been shown to reduce PMN accumulation, which is instrumental in reducing infarct size (Granfeldt et al. 2012). This was attributed to involvement of a reduction in superoxide (Granfeldt et al. 2012). Further to this, the inhibition of PMN adhesion and degranulation, as well as in the context of neutropaenia, has been shown to elicit a cardioprotective effect following I/R (Simpson et al. 1990).

1.3.7 Alteration of Ca^{2+} homeostasis

It was first described by Shen and Jennings in 1972 that myocardial reperfusion was accompanied by Ca^{2+} overload (Shen and Jennings 1972). Changes in intracellular Ca^{2+} homeostasis have since been shown to play an important role in the pathogenic outcome of reperfusion injury (Chen et al. 2006, Chen et al. 2011). Primarily, this appears to be due to increased entry of Ca^{2+} into the cytosol via specific calcium channels in the sarcoplasmic reticulum (Gao, Korthuis and Benoit 1996, Wier et al. 1997). Further to this, activation of Ca^{2+} dependent proteases, such as calpain I and troponin I, result in myofibril proteolysis and the resultant activation of signalling pathways, leading to reperfusion injury, inevitably follow (Verma et al. 2002).

Following I/R injury, there is an alteration in Ca^{2+} homeostasis which is observed as excessive levels of intracellular Ca^{2+} concentrations. This is primarily attributed to

dysfunction of the sarcoplasmic reticulum and NCX which, in the context of healthy cells, hold crucial roles in maintenance of Ca^{2+} regulation (Chen et al. 2006). The endoplasmic reticulum has been demonstrated as holding an integral role in mediating cellular Ca^{2+} levels in the context of cellular stresses such as oxidative stress as those associated with I/R (Flesch et al. 1996). Primarily these mechanisms are thought to involve the NCX (Motegi et al. 2007) (described in section 1.3.1).

Historically, increases in cytosolic Ca^{2+} as a determinant for myocardial injury following I/R were considered unimportant (Cobbold and Bourne 1984, Kim, Jin and Lemasters 2006). However, within the past two decades, the majority of studies have provided evidence that deregulation of Ca^{2+} homeostasis, thus leading to an increase in cytosolic Ca^{2+} levels, precedes irreversible myocardial injury as a consequence of I/R (Imahashi et al. 2005, Koretsune and Marban 1989).

The endoplasmic reticulum is important in the maintenance of Ca^{2+} mediated intrinsic apoptosis, through mechanisms which have originated from either an extra- or intra-cellular context (Scorrano et al. 2003). This is partially due to the activation of BH-3 only domain members of the Bcl-2 family of proteins, such as Bim and BAD (Morishima et al. 2004). Following this, upstream pro-apoptotic signalling is relayed to the endoplasmic reticulum, which produces pro-apoptotic stimuli via the mitochondria. The intracellular increases in Ca^{2+} promote opening of the mPTP due to associated mitochondrial Ca^{2+} overload, therefore leading to depolarisation and permeabilisation of the inner mitochondrial membrane and subsequently myocyte death via necrosis (due to outer mitochondrial membrane rupture) and apoptosis (due to release of pro-

apoptotic factors into the cytosol) (Kim, Jin and Lemasters 2006, Miura and Tanno 2011).

Although necrotic and apoptotic pathways following I/R injury are fully described in section 1.4, there is also potential that endoplasmic reticulum mediated increases in Ca^{2+} lead to apoptosis via a caspase-12 dependent mechanism. This occurs as caspase-12 is activated either through the intrinsic machinery of the endoplasmic reticulum, but also via calpain cleavage (as observed via increases in Ca^{2+}). Following the activation of pro-caspase-12, caspase-12 is then capable of initiating apoptosis via cleavage of caspase-9 through a mechanism which is independent from both mitochondrial apoptogen release (specifically APAF-1 and cytochrome c) and heptodimeric apoptosome formation (McIlwain, Berger and Mak 2013).

1.3.8 Altered myocardial metabolism

Despite reperfusion being a necessary process in order to permit recovery of the ischaemic myocardium, the alteration of normal metabolism following ischaemia may potently contribute to a delay in functional, myocardial recovery (Verma et al. 2002). In particular, the limitation of oxygen availability to the heart during ischaemia prevents oxidative phosphorylation and, as such, cardiac myocytes resort to anaerobic respiration in order to fulfil their high ATP demands (Weinberg et al. 2000). However, persistent lactate production, as a result of anaerobic metabolism, frequently still occurs following reperfusion due to an inability of the heart to immediately recover and revert back to aerobic respiration. This is evidenced by a 40% reduction in normal

mitochondrial pyruvate dehydrogenase activity following reperfusion, which may persist for up to 30 minutes.

Clinically, the implication of prolonged lactate production under these conditions is an increase in contractile dysfunction of the ventricles, in particular left ventricular function (Davis et al. 2012), as well as represented by an increase in infarct size (Ferreira 2010). This was demonstrated experimentally in mice as tolerance to myocardial I/R injury has been associated with a decrease in lactate dehydrogenase. The observed decrease was associated with both a reduction in infarct size and improved contractile function following reperfusion (Xiang et al. 2011). Further to this, these associations have been linked with Rho kinase (ROCK) activity (Xiang et al. 2011). Rho is a member of a small sub-family of monomeric G-proteins and can be activated by various GPCRs, including muscarinic acetylcholine receptors (mAChRs) (Lanzafame, Christopoulos and Mitchelson 2003).

In the context of the myocardium, acetylcholine has been shown to promote cardioprotective signalling, including following I/R injury, via activation of ROCK (De Sarno et al. 2005). It is therefore possible that muscarinic receptor activation by acetylcholine promotes the restoration of aerobic metabolism, which can decrease the injurious effects of anaerobic metabolism following the onset of reperfusion.

1.3.9 Gap junctional remodelling

Gap junctions are transmembrane conduits which allow direct intercellular communication between neighbour cells via linkage of their cytoplasmic

compartments (Minamino 2009). Gap junctions are primarily comprised of hexamers of connexin proteins which are, in general, preferentially and specifically expressed in various cell types and tissues, respectively (Bruce et al. 2008). Within the myocardium, gap junctions serve to assist in myocardial contraction by facilitating propagation of action potentials between cardiac myocytes (Ando et al. 2005, Yue et al. 2006). Connexin43 (Cx43) is the predominant gap junction protein in the heart, both by abundance and functional importance (Ando et al. 2005). However, there are also roles for connexin40 (Cx40) and connexin45 (Cx45) (Minamino 2009). In particular, Cx40 plays a major role in the atria by permitting myocyte-to-myocyte propagation of the action potential during atrial depolarisation (Benes et al. 2014). Genetic ablation or mutation of Cx40 proteins have shown to cause an inherited predisposition of atrial fibrillation (Chaldoupi et al. 2009). Cx45 has an indispensible function within the atrioventricular node and bundles of His (Frank et al. 2012). Cx40 and Cx43 are generally co-expressed and it is considered that the expression of Cx45 may insulate the conductive tissues from the contractile cells and fibres within the myocardium (Lo 2000). Despite the full role of Cx45 remaining elusive, it has been observed that over expression of Cx45 serves to improve pacemaker function *iv vitro*, with a possible clinical application as a synthetic biological pacemaker in the future (Tong et al. 2010).

Following I/R, loss and hypercontracture of cardiac myocytes through necrosis produces a pathophysiology, unique to the heart, known as contraction band necrosis (CBN), which, ultimately leads to sarcolemmal rupture (Shintani-Ishida, Unuma and Yoshida 2009). Evidence from studies shows that induction of hypercontracture via micro-injection of Na^+ spreads to neighbouring myocytes via gap junctional proteins

(Ruiz-Meana et al. 1999). Further to this, the chemical uncoupling of gap junctions under conditions of I/R limits CBN as myocyte-to-myocyte gap junctional communication cannot occur (Garcia-Dorado et al. 1997), however this can result in contractile dysfunction and lethal arrhythmias (Garcia-Dorado, Rodriguez-Sinovas and Ruiz-Meana 2004). More recently, ischaemia and hypoxia have been shown to promote the intracellular translocation of Cx43 to the plasma membrane at intercalated discs, thereby enhancing myocyte-to-myocyte communication, associated with increases of CBN (Shintani-Ishida, Unuma and Yoshida 2009, Zeevi-Levin et al. 2005). However, following ischaemia, total levels of Cx43 protein have been observed to decrease (Zhang et al. 2006).

It has been well documented that the quantity of gap junctions within the heart can be regulated by acetylcholine (Murray, Nickel and Gay 2009). Following hypoxia, muscarinic acetylcholine activation is also capable of restoring suppressed Cx43 levels, therefore promoting gap junctional integrity, and inhibiting lethal arrhythmias (Yue et al. 2006, Zhang et al. 2006).

Despite the evidence appearing paradoxical, acetylcholine is also capable of eliciting cardiac myocyte preservation following I/R due to its ability to activate pro-survival kinases (as described in section 1.5). In particular, the association of Cx43 with the M_3 mAChR subtype has been shown as being impaired following ischaemia (Yue et al. 2006). However, the effect of reperfusion signalling pathways on gap junctional protein or muscarinic receptor expression is yet to be elucidated.

1.4 Cell death during ischaemia/reperfusion injury

It was previously considered that myocyte death following both myocardial ischaemia and reperfusion was a uniform, necrotic, process. The necrotic pathology of cardiac myocytes was considered to involve cellular swelling, denaturation of intracellular proteins, membrane damage and subsequent membrane rupture (Buja and Weerasinghe 2010). In particular, cardiac myocyte loss by necrosis can propagate across the intercalated discs via the CBN phenomenon (Minamino 2009). However, within the past two decades, it has become apparent that following I/R injury myocytes are subjected, as with other nucleated cells, to apoptosis as well (Dewson and Kluck 2009, Kim and Kang 2010). In contrast to classical apoptosis mechanisms, which avoid inflammatory immune responses, following I/R, cardiac myocytes do not immediately fragment to apoptotic bodies for removal by phagocytes, but elicit exudative inflammation as cardiac myocytes release small quantities of chemo-attractants upon initiation of apoptosis (Buja and Weerasinghe 2010). This indicates that the mechanism of apoptosis, in the myocardium, can further contribute to myocyte loss by necrosis as well. There is now a multitude of evidence which promotes that, following myocardial I/R, myocytes are permitted to die by apoptosis, necrosis (more specifically oncosis following ischaemia) and autophagy (Buja and Weerasinghe 2010). All death processes which occur in the infarcted and reperfused myocardium have been shown to occur concomitantly (Bishopric et al. 2001).

1.4.1 Necrosis

Necrosis is the mechanism by which the majority of cells within an organ will prematurely die following disease, injury or conditions of hypoxia/ischaemia (Wang et al. 2008) and has been described as a "destructive, irreversible death process" (Searle, Kerr and Bishop 1982). Morphologically, cells and organelles swell leading to mitochondrial dysfunction which is inevitably accompanied by the depletion of ATP. Subsequent rupture of the cell leads to intracellular components leaking into the extracellular matrix and, consequently, initiating an inflammatory immune response, thereby causing further damage to cells in the immediate periphery (Majno and Joris 1995). It was previously considered that necrosis was entirely responsible for cardiac myocyte loss following I/R (Majno and Joris 1995). However, it has since been elucidated that apoptosis and autophagy also play important roles in the fate of cardiac myocytes following an ischaemic insult (Kim and Kang 2010, Zeng et al. 2014). It is currently postulated that necrosis is responsible for approximately 35-50% of myocyte loss following I/R (Buja and Weerasinghe 2010). The major cause of necrosis in the reperfused myocardium is through opening of the mPTP which initiates primary myocyte necrosis (Hausenloy, Boston-Griffiths and Yellon 2011), further described in section 1.7.

1.4.2 Apoptosis

The word apoptosis, coined by Wyllie *et al.* (Wyllie, Kerr and Currie 1980), is derived from Greek meaning "falling off". In the context of autumnal leaves from a tree, or

petals from a flower, this was related morphologically to programmed cell death. However, now there is a clearer understanding of apoptosis, to define on functional criteria appears more appropriate: “A stepwise, tightly regulated mechanism for eliminating damaged or superfluous cells without harming their healthy neighbours” (Bishopric et al. 2001). Apoptosis is regulated by complex cellular mechanisms (Figure 1.5) and tightly regulated by a myriad of receptors, organelles and proteins, including caspases (cysteine aspartate specific proteases) which represent the most important initiators, effectors and executioners in the apoptotic machinery (Cryns and Yuan 1998).

The elimination of cells via apoptosis is fundamental in the development and maintenance of tissue integrity in multicellular organisms (Twomey and McCarthy 2005). However, apoptosis is also central to a cornucopia of disease states including degenerative processes, cancer and I/R (Kroemer and Martin 2005).

In relation to the myocardium, cardiac myocytes are activated to die by apoptosis in response to a range of cellular stresses including work overload, free radical stress and I/R injury (Bishopric et al. 2001). There is evidence that, following I/R, approximately 50-65% of myocyte death occurs via apoptosis (Buja and Weerasinghe 2010), this includes both caspase dependent and caspase independent components, of which the apoptotic cascade may be mediated by the mitochondria (intrinsic pathway) or activation of cell surface death receptors (extrinsic pathway). The full extent of apoptotic pathways in the myocardium is beyond the scope of this thesis, however, the review conducted by Orogo and Gustafsson provides an excellent overview of apoptosis within the cardiovascular system (Orogo and Gustafsson 2013).

Figure 1.5: Intrinsic and extrinsic apoptosis pathways. Intrinsic (or mitochondrial dependent) apoptosis is initiated via cellular stresses (**a**), for example DNA damage or conditions of I/R. apoptosis (**b**) occurs as a consequence of ligand binding to death receptors or the action of $CD8^+$ cytotoxic T-cells. (Adapted from Twomey and McCarthy 2005, Kroemer and Martin 2005, Orogo and Gustafsson 2013) All abbreviations within the list of abbreviations.

1.4.2.1 Caspase dependent apoptosis (intrinsic pathway)

Mitochondria, frequently known as the "power house" of a cell, are present in the majority of present day eukaryotic species (Saelens et al. 2004), and are primarily responsible for providing cells with ATP through oxidative phosphorylation (Hüttemann et al. 2007). However, following apoptotic stimuli, such as increases in ROS, as observed under conditions of I/R, the mitochondria becomes a target for the initiation of apoptosis and subsequent destruction of the cell (Dewson and Kluck 2009). This pathway is regulated by downstream G-protein coupled receptor (GPCR) signalling following environmental stresses such as those during I/R (Sun et al. 2010b). Pro-apoptotic signalling converges on the mitochondrial membrane bound Bcl-2 proteins, Bak and Bax, thus resulting in their activation and oligomerisation, to form pores within the outer mitochondrial membrane (Scorrano et al. 2003). This permits the release of apoptogenic factors (such as cytochrome c, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO) and apoptosis inducing factor (AIF)) into the cytosol, thereby initiating caspase-dependent and caspase-independent apoptotic cascades (Dewson and Kluck 2009).

The release of cytochrome c and apoptosis protease activating factor-1 (APAF-1) into the cytosol, promotes the formation of the heptodimeric apoptosome whereby cleavage of pro-caspase-9 is enabled following subsequential dimerisation of cytochrome c and APAF-1. Activation of caspase-9 then promotes downstream proteolytic pro-caspase cleavage causing a caspase dependent apoptotic cascade (Dewson and Kluck 2009). This ultimately leads to DNA fragmentation, condensation of organelles, membrane blebbing and reorganisation of the cellular constituents into

apoptotic bodies which are then removed via phagocytosis, in the absence of an inflammatory immune response (as shown in Figure 1.5).

In order to alert circulating phagocytes to their presence, and hence prevent secondary necrosis, apoptotic cells secrete specific “find me” signals such as lysophosphatidylcholine, sphingosine 1-phosphate, and the nucleotides ATP and UTP during the early stages of apoptosis (Chekeni et al. 2010). These signals recruit monocytes and macrophages to the region of apoptotic cells. In order for recruited phagocytes to identify the apoptotic cells amongst the multitude of viable cells, apoptotic bodies also present “eat me” flags. This is most commonly observed as exposure of phosphatidylserine as well as alteration of glycosylation patterns on the surface of apoptotic cells as serves to advertise the presence of apoptotic cells to the macrophages in the proximity of the dying cells (Ravichandran 2011). Interestingly, despite both “find me” and “eat me” signals being non-specific for monocytes or macrophages, during phagocytosis of apoptotic bodies there is minimal neutrophil recruitment (and hence minimisation of an inflammatory response), this has been attributed to the co-release of “keep out” signals in the supernatant of apoptotic cells, in particular lactoferrin, which act as a potent cellular deterrent for neutrophil migration (Bournazou et al. 2009).

A role for p53 has also been elucidated in the context of I/R injury. p53 is a critical regulator of the cell cycle and is best known for its tumour suppressor actions whereby p53 arrests the cell cycle at the G_1/S checkpoint. This provides a mechanism where incorrect DNA sequences in proliferating cells are identified and then either repaired, or removed through apoptosis. p53 is also capable of sensing cellular stresses, such as oxidative stress and conditions of I/R and can then couple to the apoptotic cascade.

Upon such cellular stresses, cytoplasmic p53 rapidly translocates to the cytoplasmic surface of the outer mitochondrial membrane (Vaseva et al. 2012). This primarily involves recruitment of Bcl-2 proteins (such as Bak and Bax), thereby directly promoting MOMP (Green and Kroemer 2009, Vaseva and Moll 2009). However, p53 also appears capable of preventing the repressor activity of Bcl-xL, which then indirectly promotes apoptosis, as previously described (Vaseva et al. 2012). There is also recent evidence that p53 may be a critical regulator in necrosis via interaction with cyclophilin D, a key component of the mPTP (Vaseva et al. 2012).

Despite significant evidence that intrinsic and extrinsic apoptosis pathways play an instrumental role in myocyte loss following I/R injury, there has been conflicting evidence as to whether apoptosis is initiated during ischaemia, reperfusion or plays an active role in both (Foo, Mani and Kitsis 2005, Gustafsson and Gottlieb 2007).

However, further to this, both broad spectrum caspase inhibitors (eg, ZVAD-fmk) (Buja and Weerasinghe 2010) as well as capsase-3 specific (DEVD-fmk) (AL-Rajaibi et al. 2008, Mocanu, Baxter and Yellon 2000) have been shown to limit infarct size by 21-31% and 25-39% respectively. This implies an instrumental role for caspase dependent apoptosis in myocardial I/R injury.

Further to this, the Bcl-2 proteins exhibit apoptosis regulation and, depending on the protein domains present, may be pro- or anti-apoptotic. Many of the Bcl-2 proteins are thought to associate with components of the mPTP, for example the voltage dependent anion channel (VDAC), thereby not only regulating apoptosis but also playing a role in necrotic myocyte loss (Bishopric et al. 2001).

1.4.2.2 Caspase dependent apoptosis (extrinsic pathway)

In contrast to the intrinsic apoptotic pathway, the extrinsic pathway (schematically represented in Figure 1.5) is initiated by ligand binding to cell surface members of the tumour necrosis factor receptor family, known as death receptors (Lavrik, Golks and Krammer 2005), for example Fas (also known as CD95 or Apo1) and tumour necrosis factor-α receptor-1 (TNFR1, also known as p55 or CD120a) (Crow et al. 2004).

Within the cytosolic component of death receptors, there are specific death domains (DD) (Buja and Vela 2008). Upon association of ligands (for example TNF-α, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL)) with a death receptor, the ligand-receptor complex trimerises with DR4 (TNF receptor superfamily 10a). This leads to respective recruitment of cytosolic DD-containing adapter proteins (eg. Fas associated via death domain (FADD) and TNFR1 associated via death domain (TRADD)). This causes subsequent formation of a death inducing signalling complex (DISC), which permits the oligomerisation and self-cleavage of pro-caspase 8 (Foo, Mani and Kitsis 2005). This activation of the initiator caspase, caspase-8, allows downstream activation of other caspases, Bcl-2 family members and pro-apoptotic regulators, converging on cellular death by apoptosis. Caspase-8 can directly elicit a caspase-dependent apoptotic cascade via cleavage of pro-caspase-3, such that apoptosis proceeds as with the intrinsic pathway, following caspase-3 activation. However, caspase-8 is also capable of associating with the pro-apoptotic Bcl-2 family member Bid. The protease activity of caspase-8 cleaves Bid allowing a truncated fragment (t-Bid) to translocate to the mitochondria. This permits activation of the mitochondrial (intrinsic) apoptotic machinery as t-Bid interacts with Bak and Bax, enabling MOMP, therefore forming a

bridge and cross-talk between the extrinsic and intrinsic apoptotic pathways (Crow et al. 2004).

I/R has been shown to elicit activation of both the intrinsic and extrinsic apoptotic pathways (Foo, Mani and Kitsis 2005, Gustafsson and Gottlieb 2007). In particular, mutant knock-out mice studies have shown an instrumental role for both intrinsic and extrinsic apoptotic pathways in the absence of the other (Buja and Vela 2008). However the contribution of both to overall loss of myocytes by apoptosis remains as a matter of some debate.

1.4.3 Other death pathways

Autophagy is defined as a process by which lysosomes degrade proteins and organelles, such that they can be used as substrates by other cells (Crow et al. 2004), although there has been considerable discussion as to whether autophagy can be considered as a cellular death process (Yuan, Lipinski and Degterev 2003). In contrast with other cellular death processes (necrosis and apoptosis) autophagy is a mechanism by which cellular constituents are utilised to produce energy, rather than a process which is energy expensive (Glick, Barth and Macleod 2010). In the context of cellular stress (for example pathological increases in ROS following I/R), autophagy may be initiated in order to scavenge nutrients necessary for the continuation of cellular metabolism (Klionsky 2007).

However, autophagy cannot proceed indefinitely, and excessive autophagy will ultimately lead to cellular death by apoptosis (Eisenberg-Lerner et al. 2009). However,

in the context of the ischaemic myocardium, autophagy can serve to scavenge damaged organelles via mechanisms which actually serve to prevent apoptosis and protect against myocyte loss (Eisenberg-Lerner et al. 2009). Studies have also provided evidence that autophagic stimuli are enhanced during ischaemia (Calise and Powell 2013). Further to this, agents which enhance autophagy have been shown instrumental in cardioprotection and reduction of infarct size (Przyklenk et al. 2011, Sala-Mercado et al. 2010). In particular, rapamycin (which inhibits the mammalian target of rapamycin (mTOR) pathway, and consequently phosphoinositol 3-kinase and Akt activation through this mechanism) induced autophagy has been shown to promote myocyte viability following I/R by a reduction in ROS and improvement of mitochondrial function (Yang, Wang and Peng 2010).

A further cell death pathway is the contemporary notion of necroptosis, a morphology of cell death which resembles programmed necrosis (Galluzzi and Kroemer 2008), where apoptotic and necrotic pathways interact (Kung, Konstantinidis and Kitsis 2011). In the context of the cardiovascular system, myocyte loss from I/R injury was previously attributed to apoptosis and necrosis. However, the identification of programmed necrosis initiated studies which identified that myocyte death through regulated necrosis also plays an important role in I/R injury (Kung, Konstantinidis and Kitsis 2011). Primarily by the suggestion that Bax has the ability to regulate mitochondrial swelling and mPTP opening and that in Bax/Bak knockout mice, the mPTP is unable to function (discussed in more detail in section 1.7, Whelan et al. 2012).

Although this remains as a matter of some controversy, it is a novel explanation for the unique pattern of myocyte loss observed during I/R. This is especially poignant when it

is taken into consideration that, in contrast to other cell types, cardiac myocyte apoptosis often also initiates necrosis although generally apoptosis does not elicit an inflammatory response and clears dying cells and the resultant debris in the absence of necrosis.

1.5 Signalling Kinase Pathways

Within the context of myocardial I/R injury, it has been shown that the activation of protective signalling pathways is paramount to salvage functional myocytes, in the reversibly damaged myocardium, and prevent fatal myocardial injury. In particular, cascades of pro-survival kinases have been identified as instrumental in cellular protection and proliferation both within the context of healthy cells, as well as I/R. In a general sense, the cellular mechanisms which underlie normal cardiac development, differentiation and growth have been shown to be up-regulated under conditions of I/R, following endogenous administration of growth factors, which elicits cardio-protection from acute I/R injury due to recruitment of pro-survival signalling pathways, which are now referred to as the RISK pathway (Hausenloy and Yellon 2004). In particular, RISK signalling is comprised of pro-survival kinases (and their associated pathways) (Davidson et al. 2006). This includes the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, despite not comprising all pro-survival signalling cascades, the extracellular signal-regulated kinase 1/2 (Erk1/2) pathway and c-Jun N-terminal kinase (JNK) pathway. Interestingly both these pathways are of particular interest in the context of I/R injury, but also, in muscarinic signalling.

1.5.1 Phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway

Participation of the PI3K/Akt pathway in cellular growth, proliferation and survival has been frequently documented, including the importance in cellular survival of cardiac myocytes (Kuwahara et al. 2000). The activation of PI3K, by appropriate ligand or cytokine binding to cell surface receptors (frequently GPCRs), leads to downstream phosphorylation of phosphatidylinositol 4,5 biphosphate (PIP_2), which ultimately leads to the formation of phosphatidylinositol 3,4,5-triphosphate (PIP_3). A proportion of this is hydrolysed by inositol phosphatase, therefore producing phosphatidylinositol 3,4 biphosphate. PIP_3 and PIP_2 then recruit Akt to the plasma membrane and fully phosphorylate Akt at Thr-308 (within Akt's catalytic domain) and Ser-473 (at the C terminus), leading to its activation (Mullonkal and Toledo-Pereyra 2007).

Akt is one of the best described survival kinases and, as such, a multitude of cellular effects have been reported following Akt activation. In particular, cardiac myocytes are terminally differentiated and do not proliferate, therefore loss of myocytes via any cellular death mechanism, plays a crucial role in the functional outcome of the myocardium following injury, such as I/R (Miyamoto, Murphy and Brown 2009). Despite Akt substrates being distributed throughout the cell, and translocation of Akt to the plasma membrane for full activation, once phosphorylated, Akt frequently accumulates in the nucleus (Tsujita et al. 2006). This nuclear proportion of active Akt is then well situated to antagonise apoptosis via the activation p38-MAPK (mitogen-activated protein kinase) as well as regulating gene transcription (Abel and Doenst 2011). Akt can also directly promote cellular survival by ameliorating pro-apoptotic

stimuli such as apoptosis signal regulated kinase (ASK-1) and glycogen synthase kinase-3β (GSK-3β).

Within the context of the endoplasmic reticulum (ER), following ER stress and the accompanying increases in Ca^{2+}, Akt activation is sufficient to down-regulate GSK3β, thereby promoting restoration of cardiac contractile function, as well as suppression of mPTP opening (Zhang et al. 2011). Further to this, at the mitochondrial level, Akt has roles in protecting myocytes from apoptosis via promotion of Bcl-2 inhibition of Bak as well as inhibition of BH3 only domain, pro-apoptotic proteins, such as BAD, Bim and Bax (Li and Sato 2001). This prevents MOMP, therefore preventing cytochrome c and AIF release into the cytosol. Consequently, Akt is also capable of indirectly inhibiting apoptosis by the suppression of caspase activation due to the limitation on apoptosome formation. However, Akt is also able to reduce caspase-3 activation, which has been shown experimentally to limit the number of apoptotic myocytes following I/R (Su et al. 2012). Following increased cytosolic Ca^{2+} as observed during I/R, Akt can also elicit cardio-protection through the mitochondria via inhibition of mPTP opening (Miyamoto, Murphy and Brown 2009).

Despite the well documented protective effects of Akt, following myocardial ischaemia, constitutive over expression of Akt is actually capable of increasing infarct size, an observation which has been attributed to an increase in necrosis, rather than apoptosis (Nagoshi et al. 2005, O'Neill and Abel 2005).

1.5.2 Extracellular signal-regulated kinase 1/2 (Erk 1/2) pathway

MAPKs are serine and threonine kinases which regulate a variety of cellular processes, including those related to proliferation and survival. There are three main families of MAPKs, which include p38-MAPK, extracellular signal-regulated kinase (Erk1/2) and the c-Jun N-terminal kinase (JNK). Primarily, MAPKs are activated by extracellular ligand binding to receptor tyrosine kinases or G-protein coupled receptors (GPCRs) (Chen et al. 2001).

Erk1/2, mediates cell proliferation and apoptosis, in particular through the Ras-Raf-Mek-Erk signalling pathway via GPCR or RTK activation (Mebratu and Tesfaigzi 2009). Activation of Erk1/2 has been shown to promote cellular proliferation and survival and inhibit apoptosis, due to rapid phosphorylation, in response to a variety of stimuli and cellular stresses, including hypoxia (Buckley et al. 1999) and I/R in cellular models including cardiac myocytes, (Brar et al. 2000, Liu et al. 2008). The proposed mechanism for action is that Erk1/2 activation is able to prevent dephosphorylation of BAD and, subsequently, restrict caspase activation due to inhibition of MOMP and apoptosome formation (Bullard, Govewalla and Yellon 2005).

In particular, in the context of I/R and ACh, Erk1/2 has been shown as an instrumental in promoting cardio-protection due to the ability of muscarinic signalling to activate Erk1/2 and elicit, at least in part, cardio-protective properties via Erk1/2 downstream signalling (Li et al. 2011).

1.5.3 c-Jun N-terminal kinase (JNK)

c-Jun N-terminal kinase (JNK) is one of the three main subfamilies of the mitogen activated protein kinases (MAPKs). JNK is stress activated and known to play an integral role in the initiation of apoptosis in various disease states, including I/R, following its phosphorylation and subsequent activation (Zhao and Herdegen 2009). The activation of JNK includes both intrinsic and extrinsic apoptotic pathways, whereas inhibition of JNK permits the attenuation of apoptosis in several cell types and under various cellular contexts (Son, Rhee and Pyo 2006, Venugopal et al. 2007). This includes cardiac myocytes following I/R (Ferrandi et al. 2004). Further to this, more recently, JNK activation has been demonstrated to trigger both apoptosis as well as autophagy, with the consequential exacerbation of myocardial I/R injury (Xu et al. 2014).

Following activation, the cellular re-localisation of JNK to the nucleus initiates transcription of pro-apoptotic genes (Dhanasekaran and Reddy 2008). It was previously considered that this was the primary process by which JNK permits a change in cellular stability, leading to an apoptotic cascade. However, it has since been elucidated that following cellular stresses, including myocardial I/R, there is also sub-cellular re-location of JNK to the mitochondria (Wiltshire, Gillespie and May 2004). The mitochondrial localisation of JNK is advantageous for the interaction of JNK with proteins which permit MOMP and therefore, subsequent activation of intrinsic apoptotic pathways (Kim et al. 2008a, Kim et al. 2008b, Wiltshire, Gillespie and May 2004). Prevention of JNK activation, either by genetic ablation, or chemical inhibition has provided evidence, *in vivo* and *in vitro*, for protection of cardiac myocytes

following I/R injury (Chambers and LoGrasso 2011, Ferrandi et al. 2004). The activation of muscarinic receptors, in particular the M_3 subtype, following innervation by endogenous ACh, has also been shown to inhibit JNK activation (Wang et al. 2003).

1.5 RISK pathway – pro-survival kinase signalling cascade

Activation of the reperfusion injury salvage kinase (RISK) pathway has been implicated as instrumental in limiting reperfusion induced injury (Hausenloy and Yellon 2004, Hausenloy, Tsang and Yellon 2005). This pathway consists of a cascade of pro-survival kinases and included phosphatidyl-3-OH kinase (PI3K)-Akt and p42/p44 extracellular signal-regulated kinases (Erk1/2) (Hausenloy and Yellon 2004). During the early minutes of reperfusion, activation of either Akt or Erk1/2, promotes activation of downstream effectors which ultimately promoted cellular survival (Hausenloy and Yellon 2004). It is well documented that Akt and Erk1/2 activation exert an anti-apoptotic action, and the RISK pathway was elucidated in view that reperfusion injury has been shown to have both apoptotic and necrotic components (Hausenloy, Tsang and Yellon 2005).

The RISK pathway has since become a target for ischaemic and pharmacological pre- and post-conditioning (Hausenloy, Tsang and Yellon 2005). In particular, pharmacological activation of this cascade, with acetylcholine as well as many other agents and growth factors, throughout the first few minutes of reperfusion have been shown as pivotal for maintenance of tissue integrity and protection against lethal reperfusion injury, via a reduction in apoptosis (Hausenloy and Yellon 2004). Further to

this, there is evidence which supports that activation of the RISK pathway is closely associated with inhibition of opening of the mitochondrial permeability transition pore (mPTP, addressed in more detail in section 1.7) (Smart et al. 2006, Davidson et al. 2006). This provides strong evidence that the RISK pathway affords protection by both a reduction in both apoptosis as well as necrosis, shown experimentally by a decrease in infarct size (Hausenloy and Yellon 2004).

1.6 Pre-conditioning and post-conditioning

Pre-conditioning was first characterised by Murry e*t al.* in 1986 where brief periods of non-lethal ischaemia followed by reperfusion were shown to have a potent cardioprotective effect against a subsequent, prolonged and more severe ischaemic insult (Murry, Jennings and Reimer 1986). This notion has since evolved to encompass pharmacological pre-conditioning, as well as post-conditioning, whereby a cardio-protective stimulus is administered after ischaemia, but prior to reperfusion.

Post-conditioning, for myocardial syndromes, at least, represents a far more attractive strategy for tissue salvage. This is because, although pre- and post-conditioning have mechanistic similarities and share degrees of tissue salvage (Okorie et al. 2011), it is almost impossible to predict a cardiovascular event, making pre-conditioning inconceivable; whereas, due to the surgical ability to regulate reperfusion, post-conditioning is a clinically viable option (Hausenloy et al. 2004).

The cellular pathways responsible for both pre- and post-conditioning have not been fully elucidated, but shared mechanistic properties have been identified, these include

the up-regulation of signalling cascades which link to the RISK pathway (Frohlich et al. 2013) (described in section 1.5). This promotes cellular survival by inhibition of the mPTP opening and activation of the mitochondrial ATP sensitive potassium channel (mitoK$_{ATP}$).

The activation of muscarinic receptors by acetylcholine has been shown as effective in both myocardial pre-conditioning (Critz, Cohen and Downey 2005, Qin, Downey and Cohen 2003) and post-conditioning (Xiong et al. 2009, Zang, Sun and Yu 2007). Acetylcholine has been shown to be protective against I/R when used in both pre- and post-conditioning due to its ability to activate the RISK pathway (Liu et al. 2011a, Mioni et al. 2005, Yang et al. 2005, Zhao et al. 2010).

1.7 Mitochondrial permeability transition pore (mPTP)

Within recent years, the mechanisms by which I/R injury occurs have been more fully elucidated, and there is increasing evidence for the involvement of the permeability of the inner mitochondrial membrane, which results in loss of mitochondrial membrane integrity, mitochondrial swelling and ultimate rupture of the outer mitochondrial membrane (Halestrap 2009). The structure within the inner mitochondrial membrane responsible for the phenomenon is known as the mitochondrial permeability transition pore (mPTP).

First reported in 1987 (Crompton, Costi and Hayat 1987), there is now a large body of evidence to suggest the mPTP, a non-specific pore located on the inner mitochondrial

membrane, remains closed throughout ischaemia but opens at the onset of reperfusion due to increases of intracellular of Ca^{2+} and oxidative stress (Griffiths and Halestrap 1995) and represents a major determinant in myocyte fate following I/R (Shanmuganathan et al. 2005). Upon opening, within the first few minutes of reperfusion, the mPTP allows free passage of molecules (≤1.5 KDa) and solutes between the mitochondria and cytosol. The build up of osmotic pressure within the mitochondria, due to the influx of solutes, causes the subsequent rupture of the outer mitochondrial membrane.

It has been almost 30 years since the mPTP was first identified and, since then, considerable efforts have been made to fully elucidate the structure and functional mechanism which leads to opening of the mPTP. Despite considerable work, primarily with the use of knock-out animals, the exact structure of the mPTP remains elusive. The mPTP was previously considered to comprise of VDAC (voltage dependent anion channel), ANT (adenine nucleotide transferase) and cyclophilin-D, located in the outer membrane, inner membrane and matrix, respectively. It has also been suggested that there is a role for the mitochondrial phosphate carrier. However, it has since been elucidated that ANT and VDAC do not appear to be critically involved in forming the channel of the mPTP (Baines 2009) as genetic ablation of both ANT and VDAC do not prevent mPTP opening (Miura and Tanno 2011).

Despite this, the role of the ANT was suggested as integral in mPTP structure with use of ANT modulators, such as atractyloside, which induced mPTP opening, as well as bonkrekic acid, which permitted its closure (Schultheiss and Klingenberg 1984). This evidence implies that the ANT may contribute to the mPTP in a regulatory capacity, rather than being structurally essential in pore formation, however there is sufficient

evidence to provide a molecular link between the ANT and mPTP formation (Halestrap and Brenner 2003). The involvement of the VDAC as a structural component has also presented difficulties. In modified mitochondria which lack the outer mitochondrial membrane, mitochondrial swelling and subsequent rupture of the mitochondrial membranes does not occur, this implies an integral role of the outer mitochondrial membrane in the structure of the mPTP (Le-Quoc and Le-Quoc 1985). As the VDAC is capable of forming electrophysiological channels (which mimic and resemble the structure and function of the mPTP), it was presumed to be the outer mitochondrial membrane component of the mPTP. However, as with ANT knock-out studies, the mPTP has been shown to adequately function in the absence of VDAC (Javadov, Karmazyn and Escobales 2009).

The majority of research to elucidate the mPTP's structure has been concentrated on cyclophilin D since the observation that the association of cyclosporin A with cyclophilin D was capable of significantly inhibiting mPTP opening – even under conditions of moderate oxidative stress and Ca^{2+} overload. With use of cyclophilin D knock-out studies, it was described that cyclophilin D null liver mitochondria required significantly higher levels of Ca^{2+} to induce mitochondrial swelling and were resilient to CsA inhibition. However, as cyclophilin D is endogenously situated within the mitochondrial matrix, it appears unlikely that it plays a structure role in pore formation (Hausenloy et al. 2010). This gives rise to the hypothesis that although cyclophilin D is involved as a critical regulatory component of the mPTP, it is not structurally involved (Baines 2009, Elrod et al. 2010). Also, as cyclophilin D is a small, soluble matrix protein, it further rules out any structural involvement in the mPTP (Baines 2009). Further to this, mPTP opening has shown to be potently inhibited by sanglifehrin A binding to

cyclophilin D (Clarke, McStay and Halestrap 2002). This provides further evidence for cyclophilin D involvement in the mPTP as, unlike CsA, sanglifehrin A does not inhibit calcineurin activity (Shanmuganathan et al. 2005).

Upon opening, the mPTP, as shown in Figure 1.6, allows free passage of molecules ≤1.5 kDa, resulting in outer mitochondrial membrane rupture. This causes primary necrosis due to ATP depletion (Yellon and Hausenloy 2007b) which occurs via the resultant collapse of mitochondrial function after disturbance of the mitochondrial membrane potential (Crompton 1999). Increase of intracellular Ca^{2+} and oxidative stress, due to rapid pH change and increases in ROS, leads to rapid ATP depletion, thereby causing rupture of the outer mitochondrial membrane. This facilitates the influx of toxic mitochondrial proteins, e.g. cytochrome-c, into the cytosol (Halestrap 2009, Halestrap and Pasdois 2009, Hausenloy et al. 2002). Thus, myocyte death via apoptosis can also occur due to outer mitochondrial membrane rupture following mPTP opening (Halestrap 2009, Halestrap and Pasdois 2009, Hausenloy et al. 2002). However, the key determinant for initiation of intrinsic apoptosis is mitochondrial outer membrane permeabilisation (MOMP). The principle components responsible for MOMP are the multi-domain Bcl-2 family members Bak and Bax (Whelan et al. 2012). Previously, the mPTP and MOMP were considered distinct components responsible for separate cell death mechanisms (Siu et al. 2008), however, it has recently been elucidated that Bak/Bax channels play an integral role in regulation of the mPTP as the absence of Bak and Bax renders cells immune from mPTP opening (Whelan et al. 2012). This further supports the contemporary notion of necroptosis, a form of programmed, regulated necrosis (section 1.3.3), and its potential involvement in I/R injury (Galluzzi and

Kroemer 2008, Kung, Konstantinidis and Kitsis 2011). Also, many of the Bcl-2 family proteins are thought to associate with the VDAC, thereby this further indicates that the regulation of apoptosis is able to interact with the mPTP and, potentially, play a role in regulating mPTP opening (Bishopric et al. 2001).

Figure 1.6: Schematic to show the proposed structure and mechanism of the mitochondrial permeability transition pore (adapted from (Bishopric et al. 2001, Miura and Tanno 2011).

1.8 Cyclosporin A

In 1988, it was first discovered that Cyclosporin A (CsA), previously used as an immunosuppressive agent, was capable of potently inhibiting the opening of the mPTP (Crompton, Ellinger and Costi 1988). The ability for CsA to bind to cyclophilin A enables immunosuppression by the action of CsA to inhibit calcineurin. Cyclophilin D is a mitochondrial isoform of cyclophilin A (Schultheiss and Klingenberg 1984). It has since

been confirmed that CsA binds with high affinity to cyclophilin D, a key component of the mPTP, thus inhibiting binding of cyclophilin D to adenine nucleotide translocase and preventing subsequent opening of the mPTP (Basso et al. 2005). Extensive work has been carried out to characterise the cellular mechanisms by which CsA exerts this effect and the potential for its use as a cardioprotective agent (Cohen, Yang and Downey 2008, Crompton 1999, Halestrap 2009, Halestrap and Pasdois 2009, Hausenloy, Boston-Griffiths and Yellon 2011, Hausenloy et al. 2002) as it targets the mPTP (Shanmuganathan et al. 2005). One of the first reported studies was from Nazareth *et al.*, who demonstrated that CsA administration was capable of reducing I/R injury in rat ventricular myocytes via a reduction in overall necrosis (Nazareth, Yafei and Crompton 1991). This provides evidence that mPTP opening primarily contributes to myocyte loss by necrotic pathways rather than apoptosis following I/R injury.

1.9 Muscarinic acetylcholine receptors (mAChRs)

Muscarinic receptors fall into the remit of the family of G-protein coupled receptors (GPCRs). GPCRs, also called seven-transmembrane receptors, represent the most abundant and diverse family of membrane bound receptors in eukaryotic cells. GPCRs are involved in a multitude of cellular and physiological processes and represent one of the major targets for the development of new pharmacological interventions for diverse pathologies (Shultz et al. 2008), including the exploitation of mAChRs for the development of new bronchodilator therapies for conditions such as COPD.

Despite the variation of ligands, all GPCRs share a distinct, evolutionarily conserved, structure. This consists of a single polypeptide which is embedded into the plasma membrane and folded, such that, seven segments transverse the entire width of the plasma membrane (Gilchrist, Li and Hamm 2002). The extracellular portions comprise the clefts to which ligand binding occurs. Ligand binding to a GPCR induces a conformational change, which initiates downstream signalling via involvement of G-proteins (Figure 1.7). More specifically, G-proteins, also known as guanosine nucleotide-binding proteins, consist of α, β and ɣ subunits. Following ligand binding to a GPCR, dissociation of the β and ɣ subunits initiates downstream signalling (specific to the α subunit subtype). This is due to the dimeric complexes which are formed by the β and ɣ subunits (Torrecilla et al. 2007).

Figure 1.7: Activation of a generic GPCR following agonist binding. Dissociation of the β and ɣ subunits following GPCR activation confers downstream signalling pathways (adapted from Li et al., 2002).

In 1914, Sir Henry Hallett Dale published his seminal paper which identified the ability of acetylcholine to elicit two types of responses, simulated by either nicotine or muscarine (Fishman 1972). Further work differentiated acetylcholine receptors, on the basis of this observation, and they were subsequently termed nictonic or muscarinic acetylcholine receptors (nAChRs and mAChRs, respectively). It has since been elucidated that both types of acetylcholine receptors are GPCRs. In particular, mAChRs are coupled to either $G_{i/o}$ or $G_{q/11}$, depending on the subtype.

There are five known subtypes of mAChRs (Caulfield and Birdsall 1998), distributed throughout the body and across a plethora of tissues (Abrams et al. 2006) responsible for a multitude of neuronal and non-neuronal actions (Caulfield and Birdsall 1998, Caulfield and Birdsall 1998, Resende and Adhikari 2009). The M_1, M_3 and M_5 subtypes are coupled to $G_{q/11}$ and the M_2 and M_4 subtypes are coupled to $G_{i/o}$.

1.9.1 Muscarinic receptor signalling

The immediate downstream signalling of the $G_{q/11}$ subtype mAChRs is the activation of Phospholipase C (PLC) which subsequently couples to PI3K activation. This signalling converges on the endoplasmic reticulum and leads to regulation of calcium via the IP_3R located on the membrane of the endoplasmic reticulum. PLC is also able to activate PKC which leads to MAPK activation, in particular Erk1/2, and therefore promotes cell survival signalling and prevents apoptosis due to inhibition of dephosphorylation of BAD.

The M_2 and M_4 subtypes are coupled to $G_{i/o}$, this primarily couples to downstream signalling which causes down regulation of adenyl cyclase (AC) and therefore inhibition of cAMP production. However, despite these differences, $G_{i/o}$ activation can also converge on diacylglycerol (DAG) and PKC signalling to elicit protection from apoptosis via Erk1/2, but also MAPK driven DNA synthesis and translation, therefore promoting cellular proliferation (an overview of muscarinic signalling cascades is outlined in Figure 1.8).

Within mammalian pulmonary systems, the M_1, M_2, M_3 and M_4 subtypes are all differentially expressed (Haddad et al. 1999, Wessler and Kirkpatrick 2008). It is the agonist action of acetylcholine on the M_3 subtype present on airway smooth muscle which is responsible for contraction in the lungs, despite approximately 80% of the mAChRs in the pulmonary system being the M_2 subtype (Barnes 2004).

Table 1.2: Localisation and differences in mAChR receptor subtypes and the signalling cascades to which they are coupled PLC = phospholipase C, IP_3 = inositol tri-phosphate, DAG = diacylglycerol, PKC = protein kinase C, AC = adenyl cyclase. In particular, M_2 and M_3 receptors are expressed within the myocardium (adapted from Caulfield and Birdsall 1998).

In human airways, the M_3 receptors are coupled to the pertussis-toxin insensitive G-protein α-subunit ($G_{q/11}$), leading to downstream activation of PLC. In turn, this initiates the formation of DAG and inositol tri-phosphate (IP_3) from the phosphorylation of phosphatidylinositol 4,5-biphosphate. In patients with COPD, there is increased parasympathetic nervous system activity. Due to the environmental irritants, such as cigarette smoke, this induces bronchoconstriction (Costello et al. 1997). The action of ACh at the M_3 receptors in the respiratory system initiates smooth muscle contraction, however over stimulation, as observed during COPD exacerbations, leads to a limitation in airflow due to excessive contraction and spasm of the smooth muscle in

the bronchi and bronchioles (Coulson and Fryer 2003). The over stimulation of muscarinic innervations at the M_3 receptors also mediate mucus and water secretion and are, therefore, in part, accountable for the hyper-secretion of mucus in the respiratory systems of COPD patients (Mullol et al. 1992).

In comparison with this, the function of the M_2 receptors within the airways is still relatively unclear. In 1984, it was first demonstrated that functional M_2 receptors were present in the pulmonary system (Fryer and Jacoby 1998). The M_2 mAChR subtype is coupled to the pertussis toxin-sensitive G-protein ($G_{i/o}$) which, upon muscarinic activation, inhibits adenyl cyclase. However, there does not appear to be a direct role for the M_2 receptors in smooth muscle contraction. It is, instead, postulated that M_2 receptors play a regulatory role by preventing adenylate cyclase-dependent smooth muscle relaxation. For example, relaxation induced by β-adrenergic agonists (Jacoby et al. 1998).

Although M_2 receptors constitute the majority of mAChRs in the myocardium by concentration, it has been shown that M_1 and M_3 receptors are also present (Abrams et al. 2006). Subsequently, it has also been identified that acetylcholine is the neurotransmitter responsible for negative chronotropic and inotropic cardiac responses via the activation of all three cardiac muscarinic receptor subtypes present (Nathanson 2008).

In particular, it has been shown that M_3 receptors may be involved in pro-survival signalling cascades following I/R injury. It has also been demonstrated that, following ischaemia, after 60 minutes reperfusion, the expression of M_3 receptors is significantly increased, whereas the expression of M_2 is, statistically unchanged, but slightly

depressed and M_1 levels are indifferent from non-ischaemic controls (Yue et al. 2006). However, the clinical implications for this change in expression are yet to be fully elucidated. It is a short, but conserved, poly-basic region within the M_3 receptor C-terminal tail which appears to be responsible for the anti-apoptotic properties of muscarinic activation due to ACh binding (Budd et al. 2003).

Following I/R, $G_{q/11}$ activation, in response to serum and glucose deprivation and redox stress, initiates extrinsic, mitochondrial dependent apoptosis. Interestingly, this is specific to the myocardium, and may attest to why cardio-myocyte apoptosis exhibits differently from other cells. But also this is restricted to cardiac muscle as striated, skeletal muscle lacks APAF-1, and therefore cannot support cytochrome-c mediated, mitochondrial driven apoptosis (Bishopric et al. 2001).

Figure 1 [illegible] which permits downstream signalling via PLC, whereas M_2 and M_4 are coupled to $G_{i/o}$ which induces downstream signalling which leads to a reduction in cAMP. However, all receptors are able to contribute to downstream signalling which activates ERk1/2 and, therefore promote cellular proliferation and survival via prevention of apoptosis. (Adapted from Borroto-Escuela et al. 2010, Ogoda et al. 2011, Sun et al. 2011) All abbreviations within the list of abbreviations.

1.9.2 Acetylcholine

Acetylcholine (ACh) is an ancient signalling molecule expressed and synthesised in vertebrates, invertebrates, prokaryotes, plants and fungi (Resende and Adhikari 2009). There is evolutionary evidence to promote the concept that acetylcholine has been instrumental in modulating cell function since the origin of life in non-neuronal (Horiuchi et al. 2003) and, more recently in evolutionary terms, neuronal cells (Wessler et al. 2001).

ACh was the first neurotransmitter to be identified, by Sir Henry Hallett Dale in 1915, due to observations of its function in the heart following release from the vagus nerve. However, it was not until 1921 that Otto Loewi isolated and confirmed ACh as a neurotransmitter, although he originally named it "Vagusstoff" (which directly translates from the German as "stuff from the vagus nerve") (Dani and Balfour 2011). ACh is now one of the best characterised neurotransmitters and the role of ACh in neurotransmission has been extensively studied (overview in Figure 1.9).

Although acetylcholine is primarily considered as a neurotransmitter, non-neuronal cholinergic signalling in peripheral organs has now also been clearly defined (Caulfield and Birdsall 1998). From the context of the myocardium, endogenous acetylcholine has been observed to be readily synthesised, in both *in vivo* and *in vitro* experimental models. In particular, muscarinic innveration mediates smooth muscle contraction, glandular secretion and cardiac contraction force (Brodde and Michel 1999).

Figure 1.9: Muscarinic receptor neurotransmission on airway smooth muscle and nerves (adapted from Bargmann 2005, Coulson and Fryer 2003).

1.9.3 Acetylcholine and cardioprotection

It has been well documented that muscarinic activation by acetylcholine (ACh), the endogenous agonist of mAChRs, promotes cell proliferation and is cytoprotective in a number of experimental models, cell types (Budd et al. 2003, Buys et al. 2003, Critz, Cohen and Downey 2005, De Sarno et al. 2003, Krieg et al. 2002, Li et al. 2011, Resende and Adhikari 2009) and against various cellular insults (De Sarno et al. 2003, Resende and Adhikari 2009). This includes Chinese-hamster ovary, P19 cardiac myocyte cell line, neuronal cells as well as within the myocardium including conditions of I/R (Buys et al. 2003, De Sarno et al. 2003, De Sarno et al. 2005, Styles, Zhu and Li 2005). This protection is facilitated by the inhibition of apoptosis via activation of Phosphatidylinositol-3-OH Kinase (PI3K), leading to the activation of downstream targets such as protein kinase B/Akt and MAPK/Erk1/2 (overview of pathways in figure

1.10, Krieg et al. 2004). These pathways prevent activation of pro-apoptotic stimuli, eg. BH-3 only domain Bcl-2 proteins and caspase activation, and promote DNA synthesis thereby protecting the cell from apoptotic death (Budd et al. 2003, Li et al. 2011, Resende and Adhikari 2009). Acetylcholine has also been shown effective in protecting the myocardium against hypoxia induced apoptosis (Kim et al. 2008a, Liu et al. 2011a) by regulation of Bak, Bax and caspase-3. ACh has also been shown, specifically in the myocardium due to agonism of cardiac M_3 receptors by decreasing the activity of pro-apoptotic mediators Fas and p38 (Liu et al. 2013).

More recently, acetylcholine induced cardioprotection has been shown to be abolished by atractyloside, a known opener of the mitochondrial Permeability Transition Pore (mPTP). This indicates an integral role of the mPTP in acetylcholine mediated cardioprotection (Sun et al. 2010a). Further to this, it provides evidence for the involvement of RISK signalling as both G_i or G_q signalling link with downstream Erk1/2 activation. As previously described (section 1.7), there have been strong links demonstrated between the RISK pathway and mPTP opening (Davidson et al. 2006) which, have also been shown to have an association with non-neuronal ACh signalling.

Although conjecture, it is possible that inhibition of muscarinic signalling by a muscarinic antagonist under conditions of I/R, for example in a COPD patient suffering MI, could have opposing effects and lead to a reduction of cell survival in the reperfused myocardium when myocyte death is executed (Freude et al. 2000). Indeed the M_2 selective antagonist, methoctramine, has been shown to attenuate the cardioprotective effects of adenosine in *in vitro* models of I/R (Sun et al. 2011). It has also been observed that administration of 4-diphenylacetoxy-N-methylpiperidine

methiodide (4-DAMP), an M_3 muscarinic antagonist, is capable of abrogating ACh induced cardio-protection, due to loss of activation of Erk1/2 and Bcl-2. As a consequence, mPTP opening is not inhibited, which initiates cellular death via apoptosis and necrosis (Lim et al. 2007, Yellon and Hausenloy 2007a)

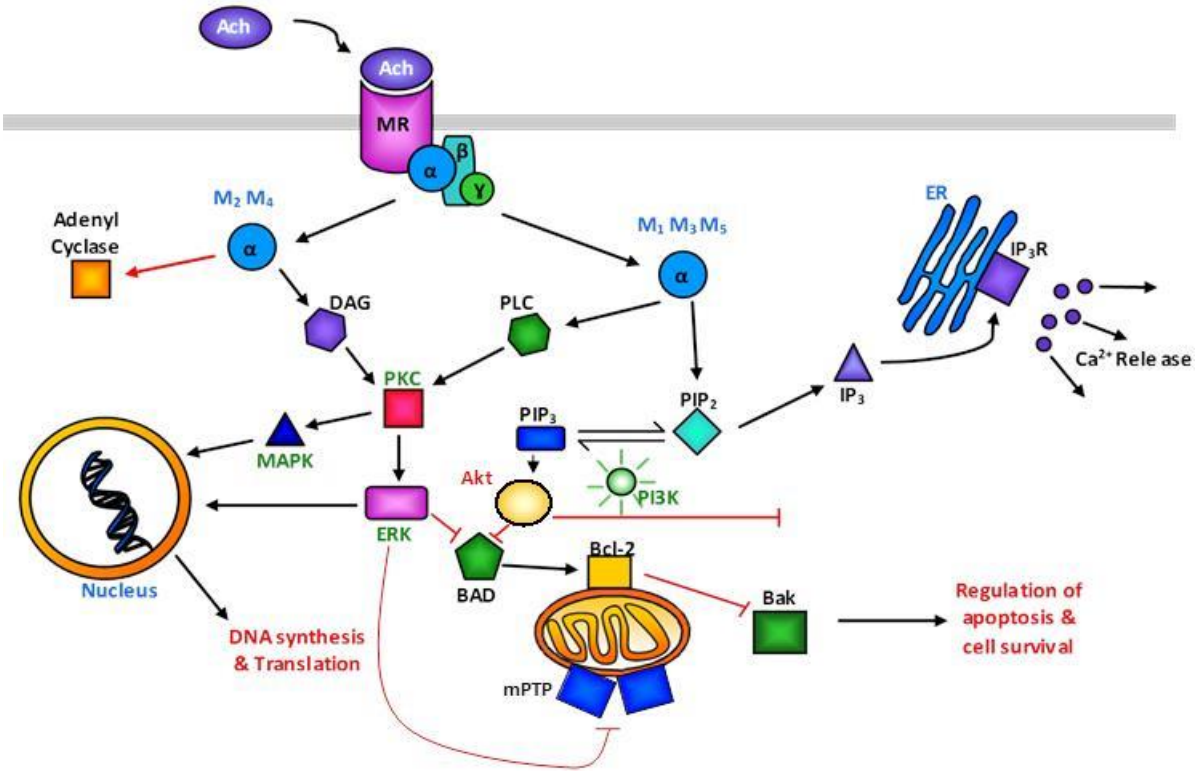


Figure 1.10: Schematic diagram to represent the convergence of muscarinic signalling pathways. Following innervations by endogenous, or exogenous, ACh, both $G_{i/o}$ (M_2 and M_4) and $G_{q/11}$ (M_1, M_3 and M_5) coupled receptors to activate ERk1/2 which, in the context of I/R, limits myocyte loss via regulation of apoptosis (through activation of Bcl-2 and inhibition of Bak) as well as necrosis via inhibition of the mPTP. The schematic clearly illustrates the involvement of components of the RISK signalling pathway and how ACh can link closure of the mPTP to RISK signalling.

1.9.4 Muscarinic Antagonists

Muscarinic antagonists are compounds which inhibit the action of acetylcholine at muscarinic receptors. Within the context of the pulmonary and cardiovascular

systems, there is an emerging, integral role for muscarinic signalling, in both the context of endogenous signalling and pharmacological interventions for disease states (as described in section 1.9.3).

Within the respiratory system, muscarinic antagonists have been instrumental in the alleviation of symptoms of bronchoconstriction, in disease states such as COPD and asthma. In the context of COPD, this occurs, primarily via antagonism of the M_3 mAChR subtype within the bronchi (Restrepo 2007).

1.9.5 Atropine

Atropine is a naturally occurring tropane alkaloid which may be extracted from members of plants from the *solanaceae* family (Gaire and Subedi 2013). In particular, it is known as a derivative from *Atropa belladonna* (Deadly nightshade) and *Madragora officinarum* (Mandrake). Atropine is a competitive antagonist for all 5 muscarinic receptor subtypes and was previously used as a bronchodilator for pulmonary pathologies due to its parasympatholytic actions in the bronchi, therefore leading to bronchodilation. This is primarily due to antagonism of the M3 subtype located on smooth muscle within the airways.

However, due to the ability of atropine to cross lipid membranes, as well as its high absorbance rate, atropine administration is responsible for many adverse side-effects including hallucinations, ventricular fibrillation, tachycardia and nausea (Szajewski, 1995). In addition to this, cumulative doses of atropine can lead to widespread paralysis which, in severe cases may lead to coma and death, due to off-target

inhibition of parasympathetically innervated organs. For these reasons, synthetic atropine derivatives, which also possess the potent anti-muscarinic action, have been developed in order to limit the systemic, adverse side effects from atropine inhalation.

1.9.6 Ipratropium

Ipratropium bromide ([8-methyl-8-(1-methylethyl)- 8-azoniabicyclo[3.2.1] oct-3-yl] 3-hydroxy-2-phenyl-propanoate, ipratropium), a non-selective, short-acting, muscarinic receptor antagonist and synthetic atropine derivative (Wood et al. 1995), has been widely prescribed for management of pulmonary conditions, primarily Chronic Obstructive Pulmonary Disease (COPD) and as adjunctive therapy for acute asthma since 1987 (Restrepo 2007). The low absorbance rate of ipratropium, especially when administered by inhalation, coupled with its poor distribution across lipid-membranes made it a preferential treatment in comparison with atropine as the numerous adverse effects due to systemic atropine absorbance were essentially eliminated (Restrepo 2007). Ipratropium is non-selective for M_1, M_2 and M_3 muscarinic receptor subtypes (Restrepo 2007). The antagonist action of ipratropium at the muscarinic receptors in the lungs (primarily M_3 subtype) inhibits acetylcholine induced vagus nerve-mediated reflexes, leading to smooth muscle relaxation and bronchodilation. This facilitates airflow by improving pulmonary function and alleviating the bronchospasm symptomatic of COPD exacerbations (Restrepo 2007, Tranfa et al. 1995).

1.10 Link between ipratropium and ischaemia/reperfusion injury

Within the past decade, despite much controversy, numerous studies have indicated cardiovascular risks associated with COPD patients receiving anti-cholinergics (Anthonisen et al. 2002, Guite, Dundas and Burney 1999, Lee et al. 2008, Macie et al. 2008, Ringbaek and Viskum 2003, Singh, Loke and Furberg 2008, Wedzicha et al. 2008). In particular, since 2008, various analyses of clinical trials have associated an increased risk in occurrence and severity of adverse cardiovascular outcomes in COPD patients, including MI, with long-term use of anti-cholinergic drugs (Ogale et al. 2010, Rabe 2010, Singh, Loke and Furberg 2008, Singh et al. 2011), indicating an increased risk of up to 52% mortality due to cardiovascular events in COPD (Singh et al. 2011). It is known that mortality by cardiovascular events in COPD patients is higher than all other causes of death and, in particular, 22% die from complications following an ischaemic event. However, despite the known co-morbidities, there have been no known pre-clinical investigations to assess the effects of anti-cholinergic compounds, such as ipratropium, in the setting of IHD or MI.

Ipratropium represents just one of the non-selective muscarinic receptor antagonists which have been indicated as instrumental in adverse cardiovascular outcomes (Dmochowski and Staskin 2005, Montuschi et al. 2013). Despite this study primarily assessing the adverse affects following I/R, with the focus on myocyte loss through either apoptosis or necrosis, there is also evidence to suggest that antagonism of cardiac mAChRs, in particular the M_2 subtype, is capable of eliciting disease through

modulation of ventricular contraction force, atrio-ventricular conduction and QT interval prolongation (Andersson, Campeau and Olshansky 2011).

Further to this, tiotropium (an M_1, M_3 selective anti-cholinergic) has been shown to limit fibroblast proliferation in the pulmonary system (Pieper, Chaudhary and Park 2007). Whilst therapeutically beneficial, this implies that muscarinic antagonism has the potential to limit proliferative processes, which, depending on cellular context, may be detrimental to tissue integrity. More recently, Shaik *et al* have demonstrated that ipratropium is capable of eliciting suicidal erythrocyte death (eryptosis) via a mechanism dependent on the stimulation of increased cytosolic Ca^{2+} activity (Shaik et al. 2012). Despite differences between eryptosis and apoptosis, which only occurs in nucleated cells, both processes can be initiated by increases in Ca^{2+} concentration, indicating that ipratropium may have the ability to also trigger apoptosis. In addition, increases in intracellular Ca^{2+} concentration are associated with conditions of I/R and the resultant, corresponding myocardial injury. This further indicates that muscarinic antagonism potentially plays a role in the exacerbation of ischaemic injury.

From an intracellular signalling perspective, muscarinic activation by endogenous and exogenous acetylcholine has shown to promote cellular survival through activation of pro-survival signalling cascades (including the RISK pathway and via clsure of the mPTP). In a clinical context, it may be the case that anti-cholinergics have the converse effect and limit these protective processes which, under conditions of IHD or I/R, may have a pathological effect.

1.11 Studies in Aging

The elderly (>60 years old) represent the fastest growing human age group globally. It is postulated that by 2020, approximately 20% of the world's population will fall into this category (Cencioni et al. 2013). The average age for a COPD diagnosis is 67 years and, in addition to this, more than 80% of patients with ischaemic heart disease are 65 years or older (Goff et al. 2007). In combination, approximately 22% of COPD and IHD patients share both morbidities (Resende et al. 2007). However, despite an increase in frequency in myocardial infarction in the elderly, as well as age as a contributor for COPD and IHD development, the cellular mechanisms and physicological differences which occur in the aged myocardium have not been fully explored.

Despite still being a matter of some contention, there is mounting evidence supporting that myocardial I/R injury is not only more common, but also, more severe in both elderly patients and experimental models using aged animals (Boengler, Schulz and Heusch 2009, Kajstura et al. 1996, Lesnefsky et al. 1994, Ramani et al. 1996). Also, despite conflicting evidence, the efficacy of ischaemic pre-conditioning does not appear to be preserved in the aged heart, as shown in *in vivo* rabbit models (Przyklenk, Li and Whittaker 2001). However, the cellular mechanisms underpinning this remain unclear.

It has also been reported that the inflammatory response, associated with I/R, is exacerbated in the aged rat heart. This is associated with significant increases in arrhythmias in comparison with young rats, and therefore poorer clinical outcomes (Liu et al. 2002, Nejima et al. 1989). During reperfusion, the timely restoration of blood

flow to the heart is imperative in avoiding fatal injury, within the senescent cardiovascular system, endothelial dysfunction has been described. This causes an exacerbation in the alteration of the restoration of coronary flow following I/R therefore leading to myocyte death (Besse et al. 2001).

Mitochondrial metabolism and oxidative phosphorylation have been shown to decrease in the aged rat heart (6 months vs. 24 months, using Fischer 344 rats), which has been attributed to a decrease in complex III and IV activity (Paradies et al. 1993) during the electron transport chain. However, the activity of complex I and II appear resilient to the aging process (Lesnefsky et al. 2001). Also, within the mitochondria, aging increases oxidative stress, even at baseline levels, prior to myocardial injury (Boengler, Schulz and Heusch 2009), this is attributed to dysfunction of the cytochrome c binding site of complex III. However, within the context of the healthy myocardium, aging does not appear to influence ROS generation (Lesnefsky et al. 2001). Furthermore, myocardial senescence, alone, appears to have a limited effect on haemodynamic function or baseline apoptosis in comparison with younger rats. However, following an ischaemic insult, there are significant increases in haemodynamic dysfunction and apoptosis activation (Kajstura et al. 1996). This is further associated with a loss of endogenous adenosine receptor signalling in the aged heart (Willems, Ashton and Headrick 2005).

Following conditions of myocardial I/R, increased severity of injury in the aged myocardium is associated with prolonged accumulation of Ca^{2+} and oxidative mediated damage (Boengler, Schulz and Heusch 2009). Previous work has provided evidence that complex III becomes damaged following I/R in the young heart (Lesnefsky and

Hoppel 2003). Also, complex III is implicated as instrumental in ROS generation during reperfusion in the adult heart, it is postulated that the dysfunction of complex III in the aged heart, contributes to enhanced oxidative stress and, consequently, myocardial injury (Lesnefsky et al. 2001).

In general, the mechanistic pathways of I/R injury have been predominantly identified, however the relative contributions of necrosis and apoptosis remain as a matter of some debate. However, despite evidence that levels of necrosis are indifferent between 16 month and 24 month old rats (Kajstura et al. 1996), it was demonstrated that left ventricular apoptosis increased by over 200% in the same age groups (Kajstura et al. 1996). Age-associated increases in Bcl-2, Bax and cytochrome c (all major components of the instrinsic apoptotic cascade) have been observed to have involvement (Liu et al. 2002, Phaneuf and Leeuwenburgh 2002). In particular, the ratio between mRNA levels of Bax and Bcl-2 is augmented, which may contribute to the observed increases in apoptosis in the aged myocardium following the challenge of I/R (Liu et al. 2002). More recently, increased levels of caspase-3 and TUNEL staining, with use of 8 week and 24 month Sprague-Dawley rats, were demonstrated and used, in conjunction with clinical trials, to confirm a correlation between increases in apoptosis and aging (Liu et al. 2011b). These studies support the hypothesis that apoptosis plays a major role in the exacerbation of I/R injury in the aged myocardium. This notion is indirectly supported by Guo *et al*. who indicated an integral role for apoptosis in post-ischaemic heart failure (Guo et al. 2008).

Under conditions of myocardial I/R, a major determinant for myocyte fate is the opening of the mPTP, which occurs within the first few minutes of reperfusion

(Hausenloy et al. 2004). As previously described (section 1.10), cyclosporin A (CsA) inhibits mPTP opening via binding to cyclophilin-D, which therefore elicits CsA mediated cardioprotection (Xie and Yu 2007). This has been widely documented and observed, as significant decreases in infarct size as well as modulation of critical mediators of I/R injury, following CsA administration. In the context of the aged myocardium, CsA is incapable of reducing infarct size. In conjunction with this, NAD^+ loss following mPTP opening was not different in the aged hearts following I/R with or without CsA administration (Liu et al. 2011b). Given that the cardioprotective effects of CsA can be inhibited by elevations of Ca^{2+}, it is possible that the inability for CsA to protect in the aged myocardium involves a similar mechanism as Ca^{2+} levels are significantly higher following I/R in aged hearts in comparison with young hearts. Also, it is possible that the observed defects within the mitochondria and increases in oxidative damage due to aging are sufficient that the mitochondria are less resilient to oxidative stress, such that CsA is unable to protect (Liu et al. 2011b).

Taken in combination, there is sufficient evidence that myocardial damage following I/R in the aged heart is more severe. From a clinical perspective, this implies that ischaemic damage in elderly patients, following myocardial infarction, may be exacerbated as a consequence of myocardial senescence. However, despite this, there is a significant lack of literature concentrating on the elucidation of the mechanisms involved in I/R in the aged heart, even though the elderly are the population most at risk for ischaemic heart disease and myocardial infarction (Valente et al. 2010).

Aging is associated with a reduction in function and expression of many hormone and neurotransmitter receptors (Brodde et al. 1998). In the context of myocardial

muscarinic acetylcholine receptors (mAChRs), there is accumulating evidence that M_2 mAChRs (the primary subtype within the myocardium) decrease in number and function following aging. The consequence of this is that endogenous acetylcholine is incapable of eliciting the negative inotropic and chronotropic effects observed in younger individuals (Brodde et al. 1998). Following I/R, this could contribute to the increases in arrhythmias which have been observed in senescent hearts. It has not been ascertained whether the cardioprotective properties of acetylcholine are abolished in the aged myocardium. Also, despite controversial evidence that elderly COPD patients require different treatments, clinical trials have not associated age dependent changes in the efficacy of anti-cholinergic compounds, such as ipratropium (Valente et al. 2010).

1.12 Experimental models for the study of myocardial ischaemia/reperfusion injury

In cardiovascular research, one major hurdle in advancing the understanding of the mechanisms and related pathophysiological manifestations of I/R injury is the availability of appropriate experimental models. Ideally, *in vivo* models which are clinically accurate to disease states observed in human patients would be used, however, practical and ethical difficulties prevent this in animal models. The most relevant models we have available are from human tissue, however, the availability of tissue is limited and both difficult and costly to obtain. In view of this, the majority of cardiovascular models are taken from healthy animals (both *in vivo* and *in vitro*) and specific protocols to mimic disease states are implemented. In this study, two main *in*

vitro experimental models, using rat heart, were used; these were the isolated perfused Langendorff heart and also a model of isolated primary ventricular rat myocytes.

1.13 Aims and objectives

The primary aim for this work is to determine whether ipratropium bromide is capable of eliciting myocardial injury, in the setting of normoxia as well as under conditions of ischaemia/reperfusion, in order to ascertain whether there is a real risk for COPD patients, with underlying IHD, receiving anti-cholinergic compounds.

Objectives:

1. To determine whether ipratropium bromide when administered under normoxic or simulated I/R conditions is capable of exacerbating myocardial damage and to identify whether the observed injury is due to necrosis or apoptosis. Further to this, to identify whether the observed injury, due to ipratropium administration, is via a direct action on cardiac muscarinic receptors.

2. To elucidate the involvement of the components of the RISK pathway, Akt and Erk1/2 in myocardial tissue following ipratropium treatment ± acetylcholine. The same experimental groups will be used to identify involvement of the stress induced kinases SAPK/JNK and the pro-apoptotic BCl-2 protein BAD, which should therefore assist in identifying the signalling pathways involved in ipratropium induced myocardial damage.

3. To identify the involvement of the mitochondria and mPTP in ipratropium induced injury using a model of oxidative stress. Further to this to assess whether the cardioprotective agent CsA is capable of attenuating the myocardial damage due to ipratropium administration.

4. To investigate whether ipratropium induced myocardial injury is further exacerbated in the aged myocardium under conditions of ischaemia/ reperfusion and oxidative stress.

Chapter 2 Materials and Methods

2.1 Materials

All drugs used (ipratropium bromide (ipratropium, Ip), atropine, cyclosporin A (CsA), Z-DEVD-FMK (DEVD) and wortmannin) were purchased from Tocris Cookson (Bristol, UK), with the exception of acetylcholine chloride (acetylcholine, ACh), which was purchased from Sigma Aldrich (Poole, UK). All drugs were dissolved, aliquotted into 1.5 ml centrifuge tubes and stored at -20^{o}C prior to experimental use. Ipratropium, atropine and acetylcholine were dissolved in ddH_2O. DEVD and Wortmannin were dissolved in dimethyl sulphoxide (DMSO), purchased from Fisher Scientific (Loughborough, UK). The final concentrations of DMSO, once diluted in the buffers, did not exceed 0.02%. This is a concentration which has previously been shown to have no effect on myocardial heamodynamic function or infarct development (Mocanu, Baxter and Yellon 2000). CsA was dissolved in ethanol, such that the final concentration of ethanol did not exceed 0.01% when used experimentally as this concentration of ethanol has also shown no adverse effects on myocardial function or infarct development (Shanmuganathan et al. 2005).

All chemicals used to prepare Krebs-Heinsleit buffer (for Langendorff studies) as well as 10% formalin were purchased from Fisher Scientific (Loughborough, UK). Both Evans blue and 2,3,5-triphenyl tetrazolium chloride (TTC), required for staining of the hearts, were purchased from Sigma-Aldrich (Poole, UK).

All salts for the preparation of modified Krebs-Ringer's buffer (required for cardiac myocyte isolation), restoration buffer and Esumi ischaemic buffer (required for cardiac

myocyte hypoxia/re-oxygenation protocol) were acquired from Fisher Scientific (Loughborough, UK), with the exception of taurine, sodium pyruvate, bovine serum albumin (BSA), HEPES, lactate, deoxyglucose and penicillin/streptomycin (all required for myocyte studies) which were purchased from Sigma Aldrich (Poole, UK). Worthington's Type II collagenase was bought from Lorne Laboratories (Berkshire, UK).

For the flow cytometry experiments, all antibodies (cleaved caspase-3, phospho-Akt and Phospho-BAD) were acquired from New England Biolabs (Ipswich, MA, USA), as were the reagents required for the Vybrant® apoptosis assay kit. For the purposes of the acetylcholine assay, a choline/acetylcholine assay kit was purchased from AbCam (Cambridge, UK). For the cardiac myocyte model of oxidative stress; HEPES, tetramethylthodamine methyl ester (TMRM) and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) were purchased from Sigma Aldrich (Poole, UK). All other reagents were purchased as before. All samples were analysed using a FACS Calibur™ flow cytometer from BD systems (St. Edmunds, Suffolk, UK).

Mini-PROTEAN® TGX™ Precast Gels (4 - 20%) and a Mini PROTEAN® II system were used for electrophoretic protein separation and purchased from Bio-Rad (Hemel Hempstead, Hertfordshire, UK). For transfer of proteins onto PVDF membranes, Trans-Blot® Turbo™ transfer packs were used in conjunction with a Trans-Blot® Turbo™ transfer system (both Bio-Rad (Hemel Hempstead, Hertfordshire, UK)). Reagents required for the preparation of solutions used for Western blotting were purchased from Fisher Scientific (Loughborough, UK) and Sigma Aldrich (Poole, UK). For the Western blotting studies, all antibodies used (phospho-Akt (Ser_{473}), phospho-Erk1/2 (Thr_{202}/Thr_{204}), phospho-SAPK/JNK (Thr_{183}/Tyr_{185}), Akt, Erk1/2, SAPK/JNK and GAPDH)

were purchased from New England Biolabs (Ipswich, MA, USA). For detection of proteins on the PVDF membranes, SuperSignal® West Femto Chemiluminescent Substrate™ (Thermo Scientific, purchased from Fisher Scientific (Loughborough, UK)) was used in order to detect target proteins in ranges as small as femtograms, to ensure maximum visualisation of proteins.

2.2 Animals

In all experiments, male Sprague-Dawley rats were used. Adult male Sprague-Dawley rats (300 g ± 50 g body weight) were either purchased from Charles River (Margate, Kent, UK) or bred in-house (Coventry University, Coventry, UK). For the aged studies, rats were purchased as adults and allowed to mature to 12, 18 or 24 months in-house (Coventry University, Coventry, UK). All animals resided under the same conditions, received humane care, a standard diet and free access to water. Procedures were carried out in accordance with the Guidance on the Operation with Animals (Scientific Procedures) Act 1986 (The Stationary Office, London, UK).

2.3 Isolated perfused rat heart (Langendorff) model

2.3.1 Principle of the technique

The mechanism of the Langendorff perfused heart model was initially proposed by Oskar Langendorff in 1897 (Skrzypiec-Spring et al. 2007) where, primarily, Langendorff

used feline hearts to deliver blood to the myocardial tissue via aortic cannulisation. It has since been used more extensively than any other isolated organ experimental model (Skrzypiec-Spring et al. 2007)and, in particular, has been vital in cardiovascular research leading to a myriad of instrumental outputs within the field (Zimmer 1998). The Langendorff isolated perfused mammalian heart model was initially based on the isolated perfused amphibian (frog) heart, which, at the time, towards the end of the 19th century, was widely used in the field of cardiovascular physiology. The principle behind the model is that the pressure of retrograde perfusion through the aorta causes the aortic valves to close, thereby providing the heart with blood (or, experimentally, physiological buffers, such as Krebs Heinsleit buffer) via the coronary arteries. Despite perfusion essentially being in reverse in comparison with a physiological setting, pressure builds up in the ventricles and therefore mimics *in vivo* diastolic conditions (Skrzypiec-Spring et al. 2007). Adequate measurements of coronary flow may be taken directly as the volume of perfusate leaving the right atrium, is indicative of the rate of perfusate passing through the heart. Under correctly controlled conditions, the Langendorff perfusion system enables cardiac function to be maintained for several hours, during which time studies to identify the effects of gene alteration, novel therapies and, in the context of this investigation, myocardial injury due to ischaemia/reperfusion were able to be conducted (Bell, Mocanu and Yellon 2011, Skrzypiec-Spring et al. 2007).

The apparatus used consisted of reservoirs of physiological buffers which perfused the heart at a constant rate and pressure, flowing through heated coils, to ensure the perfusate was at 37 ^{o}C when it reached the heart. In order to ensure adequate oxygen

consumption of the cardiac tissue could occur, all buffers were saturated with oxygen (Figure 2.1).

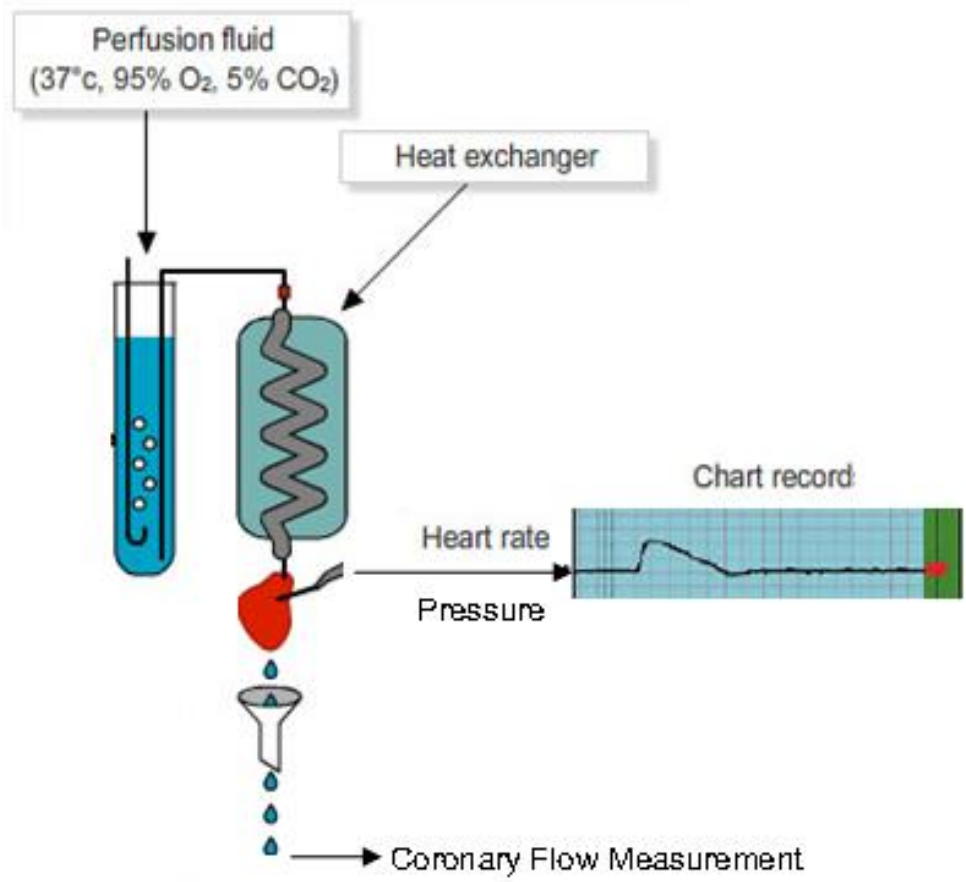


Figure 2.1: Schematic diagram of Langendorff apparatus used for isolated perfused rat heart experiments. Oxygenated, physiological buffer passes from the reservoir through a glass heated coil (at 37 ^{o}C) for retrograde perfusion of the cannulated heart, via the aorta. The heart is further maintained at 37 ^{o}C with use of a heated organ chamber. Insertion of a latex, fluid filled balloon, connected to a pressure transducer and computer allows for regular measurement of haemodynamic parameters. Coronary flow recordings may be taken directly from the measurement of the volume of coronary effluent.

2.3.2 Basic protocol

Rats were sacrificed by cervical dislocation prior to excision of the heart. The heart was immediately placed in ice cold Krebs-Heinsleit buffer (1.18 x 10^{-1} M NaCl, 2.5 x 10^{-2} M

$NaHCO_3$, 4.8 x 10^{-3} M KCl, 1.2 x 10^{-3} M $MgSO_4$, 1.2 x 10^{-3} M KH_2PO_4, 1.2 x 10^{-2} M glucose, 1.7 x 10^{-3} M $CaCl_2 2H_2O$, KHB). The aortic arch was removed to allow cannulation onto Langendorff apparatus (Figure 2.2) and retrograde perfusion with KHB (maintained at 37 ^{o}C, pH 7.4 and saturated with 95% O_2 5% CO_2). Cannulated hearts were also maintained at 37 ^{o}C with use of a heated, water-jacketed organ chamber.

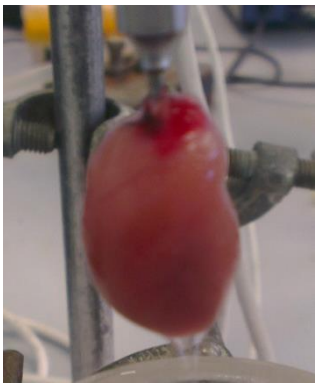


Figure 2.2: Representative image showing an adult Sprague-Dawley rat heart mounted onto the Langendorff set-up, prior to left atriotomy. The heart was cannulated by the aorta for retrograde perfusion with Krebs Heinsleit Buffer, saturated with 95% O_2 and 5% CO_2.

2.3.3 Determination of haemodynamic function

Following mounting on the Langendorff apparatus, the left atrium was removed, allowing insertion of a fluid-filled latex balloon, inflated to constant diastolic pressure of 5 - 10 mmHg, into the left ventricle. A calibrated, physiological pressure transducer connected to a PowerLab® (AD Instruments Ltd. Chalgrove, UK) via a bridge amp, connected to a computer, facilitated the regular measurement of haemodynamic function. Left ventricular developed pressure (LVDP, mmHg) and heart rate (HR, bpm) were extrapolated from a computerised trace with use of LabChart® (version 6)

software developed by AD Instruments Ltd. (Chalgrove, UK) (Figure 2.3). Coronary flow (CF, ml.min^{-1}) was measured by collecting the perfusate at regular time intervals. Measurements of all three hemodynamic parameters were taken every 5 minutes during stabilisation and ischaemia and every 15 minutes throughout reperfusion.

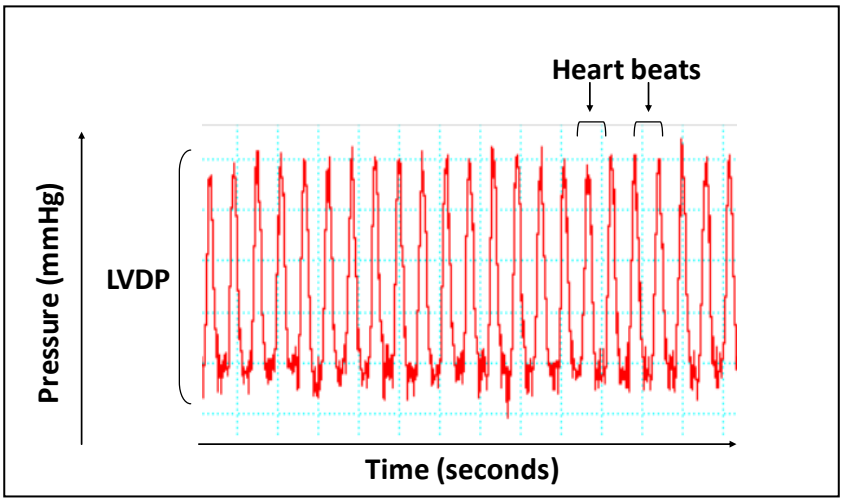


Figure 2.3: Representative trace from LabChart® 6 showing Langendorff perfused rat heart haemodynamic function. As indicated, left ventricular developed pressure (LVDP) was represented as the peak-to-peak amplitude of the trace and the length of time for one heart beat as the wavelength, from which heart rate (bpm) was calculated. Data acquisition was every 5 minutes for the first 55 minutes and every 15 minutes for the last 120 minutes of the protocol.

2.3.4 Ischaemia/reperfusion protocol

Following 20 minutes stabilisation, ischaemia was induced via insertion of a hooked 6-0 silk surgical suture under the left descending coronary arteries. The ends of the thread were passed through a plastic tube to form a snare (Figure 2.4). The snare was tightened to induce regional ischaemia in the left ventricle, for 35 minutes. Loosening of the tubes, thereby releasing the threads, initiated reperfusion as this permitted the restoration of the flow of KHB to the left ventricle. In studies subjected to the ischaemia/reperfusion (I/R) protocol, reperfusion lasted 120 minutes.

Figure 2.4: Representative image showing a surgical suture inserted around the left descending coronary arteries. The thread was passed through two plastic tubes in order to form a snare. This was subsequently tightened in order to ligate the coronary arteries, thereby initiating regional ischaemia in the left ventricle.

2.3.5 Reperfusion studies

At the onset of reperfusion, Krebs Heinsleit buffer was exchanged for Krebs Heinsleit buffer containing appropriate drug concentrations (described in respective experimental chapters). The hearts were subsequently perfused with KHB + drug(s) throughout reperfusion until Evans blue and TTC staining took place. I/R control hearts were perfused with KHB throughout the entire experimental protocol. Figure 2.5 schematically represents the three major isolated perfused rat heart (Langendorff) protocols used throughout the studies.

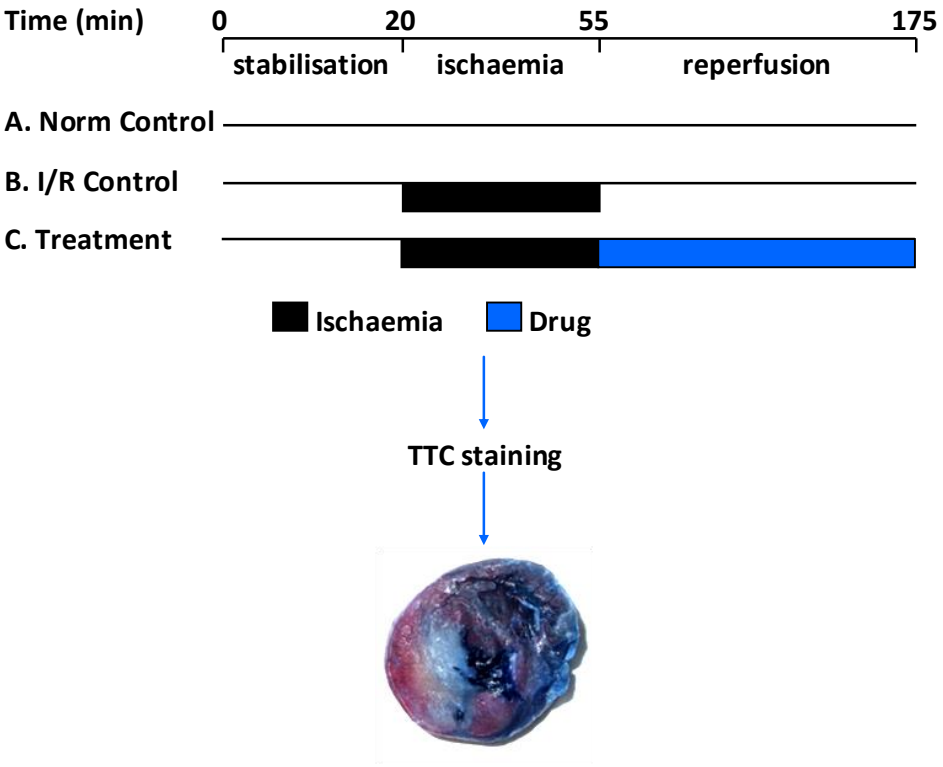


Figure 2.5: Isolated perfused rat heart protocol to show where ischaemia, reperfusion and drug administration occurred. **A.** Normoxic (Norm) control, hearts perfused for 175 minutes with KHB. **B.** Ischaemia/Reperfusion control, following 20 minutes stabilisation, regional ischaemia induced for 35 minutes followed by 120 minutes reperfusion – perfused with KHB for all 175 minutes. **C.** Treatment groups, hearts perfused with KHB for 20 minutes reperfusion and 35 minutes regional ischaemia and then with KHB containing the appropriate drug concentrations for 120 minutes reperfusion.

2.3.6 Evans blue and 2,3,5-triphenyltetrazolium chloride analysis

To delineate the ischaemic from the non-ischaemic area, at the end of reperfusion the coronary arteries were re-ligated and the heart perfused with a 0.25% Evans blue saline solution. This enabled visual differentiation between the non-ischaemic area (which stained dark blue) and the ischaemic area, which did not stain (Figure 2.6).

Figure 2.6: Representative image to show Evans blue staining following re-ligation of the coronary arteries at the end of reperfusion. Evans blue stains the non-ischaemic region of the myocardium in order to differentiate between the viable (non-ischaemic, which stains blue) and risk (area of the heart subjected to ischaemia/reperfusion protocol, which does not stain with Evans blue) zone.

The hearts were immediately placed at -20 ^{o}C until frozen and then transversely sliced into 2 mm thick sections and incubated at 37 ^{o}C in a phosphate buffer solution containing 1% 2,3,5-triphenyltetrazolium chloride, for preservation and development of the staining, hearts were placed in a 10% formalin solution for 4 hours before analysis. After staining, the viable tissue in the ischaemic area appeared red (tetrazolium positive) distinguishing it from the infarct tissue which was pale and white (tetrazolium negative). The heart slices were compressed between two Perspex sheets. The viable, risk (tissue subjected to ischaemia but viable at the end of the experiment) and infarct (tissue subjected to ischaemia which was dead at the end of the experiment) tissue were traced onto acetate film (Figure 2.7). The process was blinded, to avoid bias, and respective hearts were traced onto acetate by a colleague who had no knowledge of the experimental groups. Computerised planimetry (using Image Tool as developed by the University of Texas Health Science Centre at San Antonio,

UTHSCSA) allowed the percentage of infarct tissue within the ischaemic area to be calculated (I/R%).

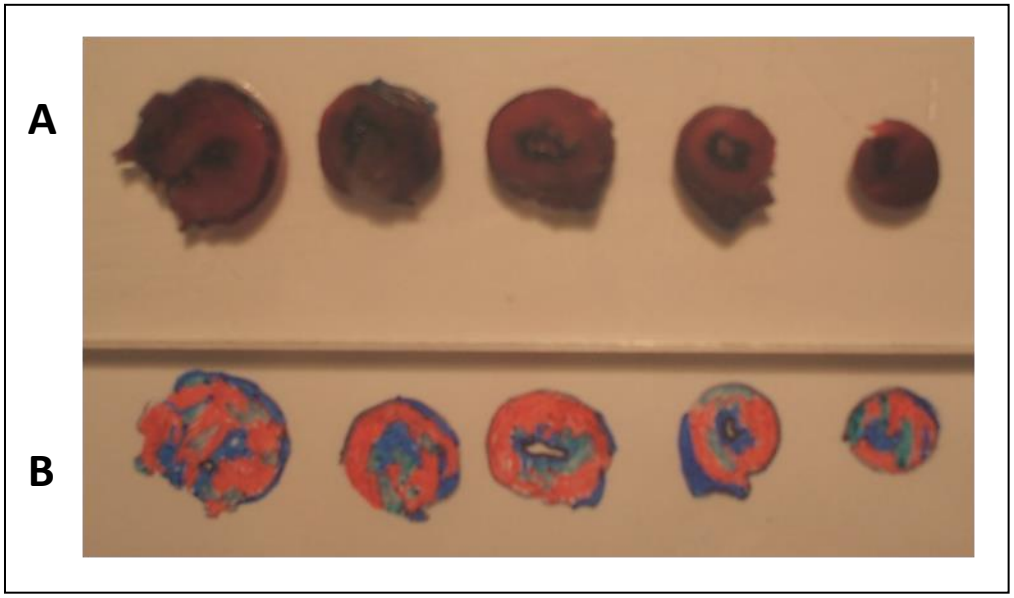


Figure 2.7: Representative heart slices following Evans blue and TTC staining. **A.** Shows heart slices compressed between two Perspex sheets (blue tissue = viable, red = risk and pale/white tissue = infarct). **B.** Shows slices traced onto acetate film, prior to computerised planimetry (blue = viable, red = risk and green = infarct).

2.3.7 Area at risk analysis

In order to determine adequate ischaemia/reperfusion protocol had been followed, the area at risk (AAR%) percentage of all hearts subjected to the Langendorff I/R protocol was calculated (data not included in this thesis). Hearts where the AAR% exceeded 45% or was less than 30% of the ventricular volume were excluded.

2.4 Adult rat cardiac myocyte isolation

Adult rat myocytes were isolated by enzymatic digestion of the extracellular matrix. Following sacrifice by cervical dislocation, rat hearts were quickly excised and mounted on a modified Langendorff apparatus for retrograde perfusion, at 7 $ml.min^{-1}$, with a modified Krebs-Ringer's buffer (KRB) (2 x 10^{-2} M $NaHCO_3$, 1.16 x 10^{-1} NaCl, 1.2 x 10^{-3} M KH_2PO_4, 5.4 x 10^{-3} M KCl, 4 x 10^{-4} M $MgSO_4.7H_2O$, 1.2 x 10^{-3} M glucose, 2 x 10^{-2} M taurine, 5 x 10^{-3} M sodium pyruvate, oxygenated with 95% O_2, 5% CO_2, pH 7.4 and maintained at 37 ^{o}C). After 2 minutes perfusion or until the heart ceased contracting and all blood had been washed out, the buffer was switched to KRB supplemented with 0.075% Worthingtons type II collagenase and 4.4 x 10^{-6} M $CaCl_2$, which formed the digestion buffer. Hearts were perfused with the digestion buffer for 7 minutes prior to removal from the apparatus and separation of the ventricles from the atria and vascular tissue. The ventricular tissue was then minced and further incubated in the digestion buffer until enzymatic dissociation was complete. Myocytes were filtered through a nylon mesh, washed with 15 ml restoration buffer (1.16 x 10^{-1} M NaCl, 2.5 x 10^{-2} M $NaHCO_3$, 5.4 x 10^{-3} M KCl, 4 x 10^{-4} M $MgSO_4.7.H_2O$, 1 x 10^{-2} M glucose, 2 x 10^{-2} M taurine, 5 x 10^{-3} M pyruvate, 9 x 10^{-4} M $Na_2HPO_4.12H_2O$, 1% BSA and 1% Pen-Strep, at 37 ^{o}C and pH7.4, RB) and maintained, in RB at 37 ^{o}C whilst the Ca^{2+} concentration was gradually increased to 1.13 x 10^{-3} M to avoid calcium overload. This was via administration of 5, 34 µl, doses of 1 x 10^{-1} M $CaCl_2$. Myocytes were subsequently transferred to a 90 mm Petri dish and maintained at 37 ^{o}C prior to experimental use.

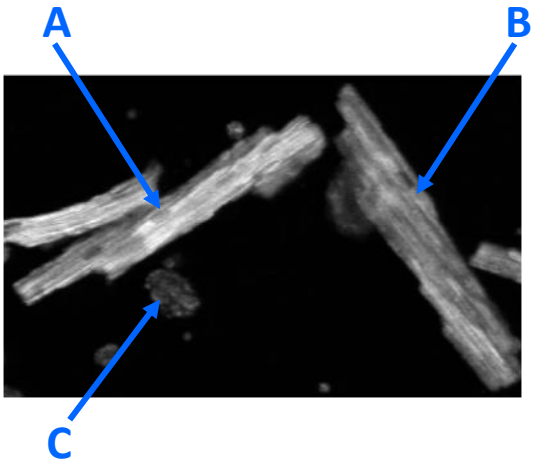


Figure 2.8: Image taken from the confocal microscope showing adult rat ventricular myocytes following isolation via collagenase digestion, **A** and **B** delineate viable, striated, cardiac myocytes, whereas **C** shows a myocyte following hypercontracture and subsequent necrotic death (magnification x40).

2.4.1 Hypoxia and re-oxygenation protocol

Cardiac myocytes were incubated, in the same media, at 37 ^{o}C for 5 hours. Myocytes were retrieved from the incubator and both yields amalgamated into one 50 ml centrifuge tube. The cells were centrifuged at 500 rpm for 2 minutes after which the supernatant was removed and disposed of using a sterile pastette. The cells were re-suspended in 10 ml RB (at 37 ^{o}C) and diluted to an adequate volume to allow for 1 ml per experimental group. 1 ml aliquot was removed and placed in a well on a 24 well plate for the normoxic control. This was placed in the incubator at 37 ^{o}C for the duration of hypoxia and re-oxygenation. The remaining myocytes were centrifuged, supernatant carefully decanted and re-suspended in 15 ml Esumi Ischaemic buffer (1.2 x 10^{-2} M KCL, 4.9 x 10^{-4} M $MgCl_2$, 9 x 10^{-4} M $CaCl_2$, 4 x 10^{-3} M HEPES, 1 x 10^{-3} M Deoxyglucose and 2 x 10^{-2} M lactate) (Esumi et al. 1991). The cells were transferred to

a Petri dish and placed in a hypoxic chamber, pre-heated to 37 ^{o}C. The atmospheric air in the chamber was then removed by use of a suction pump and replaced with argon containing 5% CO_2. The myocytes remained under hypoxic conditions for 2 hours, after which re-oxygenation was initiated by removal of the myocytes from the hypoxic chamber and transferring myocytes to a 50 ml centrifuge tube and spun at 500 rpm for 2 minutes. The supernatant was removed, discarded and cells re-suspended in RB, to provide 1 ml per experimental group, giving the same concentration of cells in each well as the normoxic control. Cells were stored at 37 ^{o}C throughout re-oxygenation and re-oxygenation lasted for 4 hours before experimental use of myocytes.

2.4.2 Incubation with drugs at the onset of re-oxygenation

During centrifugation, concentrations of ipratropium, atropine, ACh and CsA were prepared in restoration buffer. A single 10 µl aliquot of each concentration was placed into its own well in a 24 well plate containing the normoxic control. The hypoxic myocytes were plated out at 1 ml per well in the 24 well plate. All drugs were evenly distributed throughout the cell suspension by gentle agitation using a pipette. Myocytes were then subjected to re-oxygenation for 4 hours before undergoing Fluorescence Activated Cell Sorting Analysis (FACS), using a FACS Calibur™ flow cytometer, to ascertain the proportions of live, apoptotic and necrotic cells as well as the apoptosis markers caspase-3, Akt and BAD.

2.5 Acetylcholine assay

Choline/acetylcholine assay kit (Abcam, UK, catalogue no. ab65345) was used to determine cell lysate acetylcholine levels in myocytes under normoxic conditions and those subjected to the H/R protocol. The kit consisted of choline assay buffer, a choline probe, choline enzyme mix, acetylcholinesterase and a 5 µmol choline standard. The assay was carried out in accordance with the manufacturer's instruction (Abcam, UK). Briefly, working stock solutions were prepared whereby acetylcholinesterase and choline enzyme mix were separately dissolved in 220 µl choline assay buffer and the choline standard was dissolved in 100 µl choline standard buffer. Following cardiac myocyte isolation and hypoxia/re-oxygenation protocol (where necessary), cells were centrifuged briefly, counted via haemocytometer, and re-suspended in choline assay buffer to give 1 x 10^6 cells per well when plated in a 96 well mircoplate. The choline standard solution was used to prepare a standard curve as follows (table 2.1).

Choline (0.5 nmol/µl) volume (µl)	Choline assay buffer (µl)	Volume in well	End [Choline] in well
0	150	50 µl	0 nmol/well
6	144	50 µl	1 nmol/well
12	138	50 µl	2 nmol/well
18	132	50 µl	3 nmol/well
24	126	50 µl	4 nmol/well
30	120	50 µl	5 nmol/well

Table 2.1: Volumes of provided reagents used to prepare the calibration curve required for extrapolation of acetylcholine concentrations in cardiac myocyte lysates in normoxic conditions and following hypoxia/re-oxygenation protocol.

Standards were plated out in duplicate in the 96 well plate. The reaction mix was prepared to measure total choline and free choline as described in the table below (Table 2.2).

Reagent	Reaction mix (for total choline)	Reaction mix (for free choline)
Choline assay buffer	44 µl	46 µl
Choline probe	2 µl	2 µl
Acetylcholinesterase	2 µl	0 µl
Choline enzyme mix	2 µl	2 µl

Table 2.2: Volumes of provided reagents used in order to prepare reaction mixes to ascertain levels of total and free choline in the standards (used to produce the calibration curve) and in myocyte lysates, in order to ascertain concentrations of acetylcholine levels in adult rat ventricular myocytes.

50 µl of reaction mix was added to all wells (choline standards, acetylcholine control and myocytes), mixed and incubated in the dark for 30 minutes at room temperature (Figure 2.9, **A**). Colorimetric detection occurred via measurement on a microplate reader at OD 570 nm. Standard curves were plotted (Figure 2.9, **B**), which enabled extrapolation of the concentration of acetylcholine in the myocyte lysates.

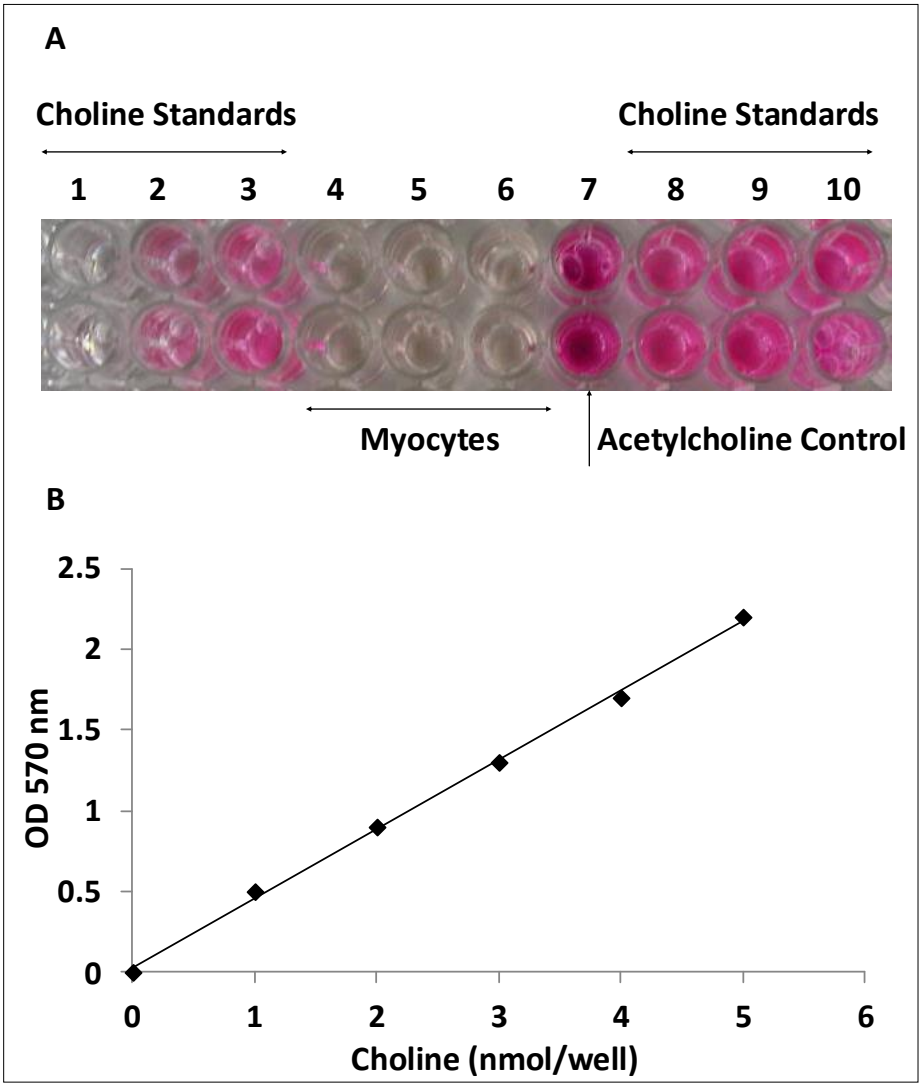


Figure 2.9: A. Representative 96-well plate showing the acetylcholine assay. Wells 1, 2, 3, 8, 9 and 10 show choline standards (0, 1, 2, 3, 4 and 5 x 10^{-9} M, respectively), used to create the standard curve. Wells 4, 5 and 6 show adult ventricular cardiac myocytes (1 x 10^{-6} cells.ml^{-1}). Well 7 shows acetylcholine, positive control (1 x10 $^{-6}$ M of acetylcholine). **B**. Representative standard curve extrapolated from the OD at 570 nm of the choline standards, expressed as a mean of triplicates (n = 4).

2.6 MTT assay

2.6.1 Principle of the technique

3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (thiazolyl blue tetrazolium bromide, MTT) is a yellow solid which, when reduced by NAD(P)H-dependent oxidoreducatse enzymes, in particular mitochondrial succinate dehydrogenase, forms a purple formazan product. Eukaryotic cell membranes are permeable to MTT, which then localises in the mitochondria and is therefore exposed to mitochondrial succinate dehydrogenase. Following lysis of cells, the formazan becomes evenly distributed throughout the media and, thus, can be measured colorimetrically via spectrophotometry or microplate reader at OD 450 nm (Weyermann, Lochmann and Zimmer 2005). It is generally accepted that the depth of colour due to MTT reduction is directly proportional to the number of cells undergoing oxidative phosphorylation, and is therefore frequently used to ascertain cellular viability. Previous work has shown the MTT assay as a suitable means of determining cellular toxicity following exposure to various compounds and, in particular, has been used to assess cardiac myocyte viability (Arstall et al. 1999, Fu et al. 2007).

2.6.2 MTT assay protocol

Ventricular myocytes were counted via Nucleo-counter (Chemometec, Sartorius, Surrey, UK). This was confirmed by haemocytometer prior to re-suspension in Esumi ischaemic buffer (Esumi et al. 1991), and exposure to 2 hours hypoxia. To initiate re-

oxygenation, myocytes were randomly assigned to drug treatment groups and re-suspended at 1 x 10^6 cells.ml^{-1} in drug treated restoration buffer (ipratropium, CsA, ACh and ipratropium ± CsA or ACh). 1 x 10^{-5} myocytes (100 µl of myocyte suspension) were aliquotted per well in a 96 well microplate. Control wells were also set up whereby replicates of 100 µl of restoration buffer (with or without drugs) were produced and aliquotted into the 96 well plates (representative plate shown in Figure 2.10). Re-oxygenation lasted 2 hours before all wells were treated with 20 µl MTT (5 mg.ml^{-1}, in PBS) and incubated, in the dark, for a further 2 hours. 100 µl lysis buffer (20% SDS in 50% N-N-Dimethylformamide) was administered to all groups and further incubated on an orbital shaker, in the dark, overnight, prior to colorimetric analysis at OD 450 nm via use of a microplate reader.

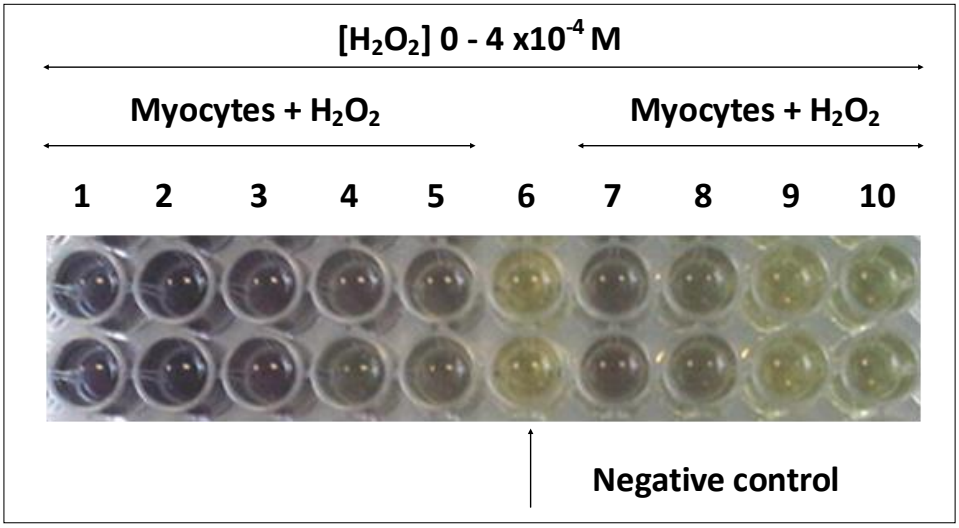


Figure 2.10: Representative 96-well plate showing MTT assay showing adult rat ventricular myocytes following H_2O_2 treatment. Wells 1-5 and 7-10 show myocytes treated with increasing concentrations of H_2O_2 from 0 – 4 x 10^{-4} M, from left to right. The depth of purple product decreases with decreased call viability, indicative of less mitochondrial dehydrogenase activity. Lane 6 shows the background control which contains all reagents, but no myocytes.

In order to analyse the data, the mean absorbance of the negative control was subtracted from the mean absorbance of the respective treatment group and represented as the percentage change of MTT reductase activity, normalised to the untreated positive control. To confirm the method, increasing concentrations of hydrogen peroxide (H_2O_2) (from 0 – 4 x 10^{-4} M) were used to create a curve indicative of cellular cytotoxicity (Figure 2.11).

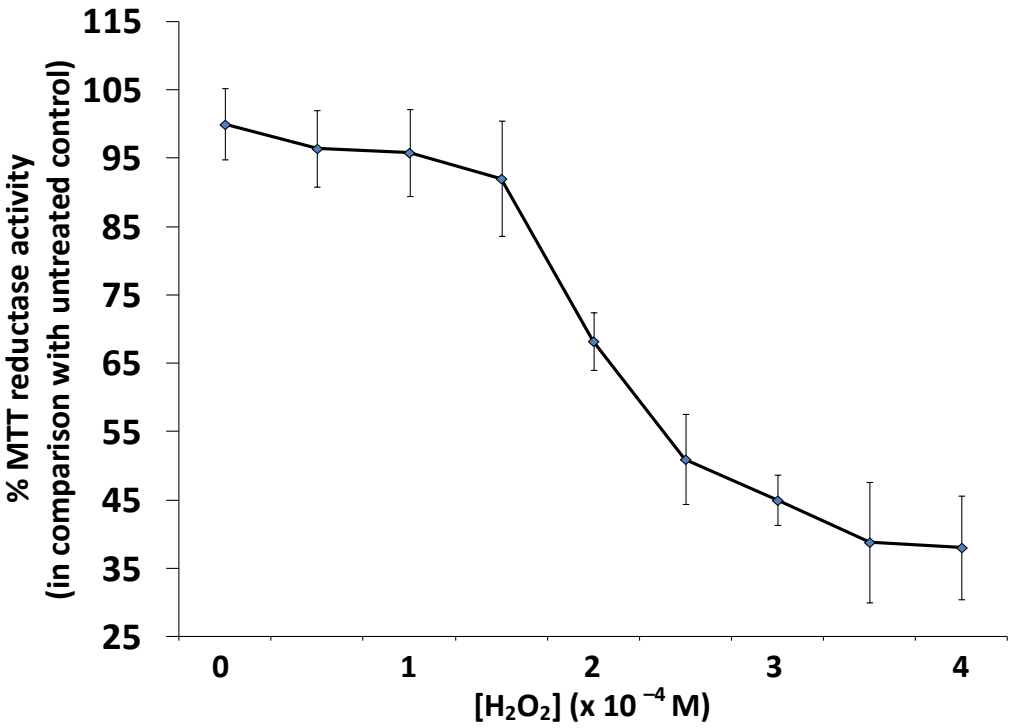


Figure 2.11: Representative scatter graph showing adult rat ventricular myocytes following H_2O_2 treatment, used to verify the protocol. Increases in H_2O_2 concentration lead to a dose responsive decrease in myocyte viability, as indicated by a decrease in the normalised MTT activity, compared with the un-treated cardiac myocytes. Values are mean ± SEM, n=6.

2.7 Flow cytometric studies

2.7.1 Principle of the method

The underpinning principles of flow cytometry were first employed in a laboratory in 1953 by Wallace H. Coulter. Flow cytometry is now a frequently utilised technique where protein expression and characteristics of cells can be ascertained by passing cell suspension through electronic detection apparatus. In particular, the equipment, known as a flow cytometer, is equipped with a laser, which illuminates particles or fluorochromes coupled to cells via specific antibodies. Cells can be labelled with highly specific antibodies which are either coupled, or secondarily stained, to fluorochromes. The levels of light are detected by the flow cytometer and converted to an electronic signal which passes to a computer system to allow quantitative analysis with use of computer software. The relative fluorescence values obtained corresponds to the levels of the labelled proteins. In this work, flow cytometry was implemented to assess proteins specific to viable, necrotic and apoptotic cells, as well as caspase-3, Akt and BAD.

2.7.2 Preparation for fluorescence activated cell sorting (FACS) analysis: cell death assay

The following protocol was used in order to discriminate the live, apoptotic and necrotic myocytes within a population. The exact percentages of each were then quantified by flow cytometric analysis using a FACS Calibur™ flow cytometer.

This was achieved using Vybrant® apoptosis assay kit with Allophycocyanine Annexin V, C_{-12} Resazurin and SYTOX® Green Stain. The apoptotic myocytes were identified via Allophycocyanine Annexin V which binds with high affinity to phosphatidylserine (PS), a protein expressed on the outer leaflet of the plasma membrane of apoptotic cells. C_{-12} Resazurin is reduced by viable cells, therefore measurement of this protein allows for live cells to be identified. Finally, the population of necrotic cells present are identifiable as SYTOX® green stain binds specifically to cellular nucleic acids present in necrotic cells.

A 1 x Annexin V buffer was prepared by diluting 4 ml 5 x Annexin binding buffer (5×10^{-2} M) HEPES, 7×10^{-1} M NaCl, 1.25×10^{-2} M $CaCl_2$, pH 7.4) in 16 ml ddH_2O. The three fluorochromes were prepared as stated in the protocol. A 5×10^{-5} M working stock of C_{-12} resazurin (Component B) was achieved by making a 5% solution of 1×10^{-3} M C_{-12} Resazurin stock dissolved in Dimethylsulfoxide (DMSO). A 1×10^{-6} M SYTOX Green working stock (Component C) was prepared by producing a 10% solution of 1×10^{-5} M SYTOX Green stock in 1 x Annexin V buffer, and then stored at -20 ^{o}C. Allophycocyanine Annexin V (2.5×10^{-2} M HEPES, 1.4×10^{-1} M NaCl, 1×10^{-3} M EDTA, pH 7.4, plus 0.1% bovine serum albumin (Component A)) did not require dilution before use.

1.5 ml Eppendorf centrifuge tubes were labelled for all concentrations of ipratropium bromide used and normoxic and H/R controls. Myocytes were taken from the incubator and removed from the 24 well plate into their respective 1.5 ml centrifuge tube. Cells were centrifuged at 500 rpm for 2 minutes before washing with 300 µl 1 x Annexin V buffer and spinning again at 500 rpm for 2 minutes. The supernatant was aspirated and discarded. 1 µl of, both components, B and C, 1.5 µl of component A and

100 µl of 1 x Annexin V buffer was added to each tube and lightly agitated to re-suspend the cells and distribute the dyes. After this, all tubes containing cells were wrapped in foil, to avoid loss of fluorescence, and incubated at 37 ^{o}C for 15 minutes. When the incubation period had elapsed, 400 µl of 1 x Annexin V buffer was added to each tube and the cells re-suspended, finalising the preparation for FACS analysis.

The fluorochromes used in this kit were deemed to be appropriate as the excitability and emission wavelengths differ on all three fluorochromes and their emission peaks do not overlap. Therefore, when analysed on the FL-2 and FL-4 wavelengths, three distinct populations, representing live, necrotic and apoptotic cells which are easily distinguishable from one another can be seen. Thereby giving results which clearly showed how many cells in the population were live, necrotic or apoptotic. The software (Cell Quest®) enabled this as quadrants were plotted into which the different populations fell, as illustrated below (Figure 2.12). The software was setup to count the first 10,000 events, therefore counting cells in preparation for FACS analysis was not necessary. The protocol used was according to the manufacturer's instructions (Invitrogen, Paisley, UK).

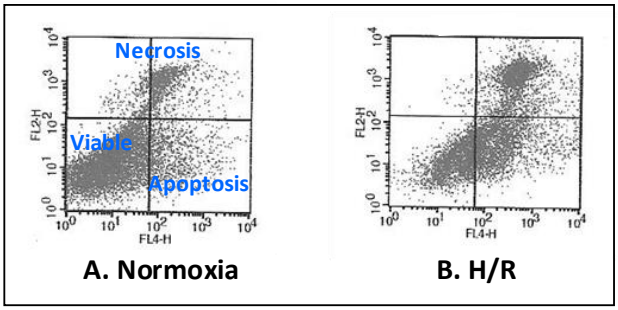


Figure 2.12: Representative flow scattergrams showing the different quadrants for viable, apoptotic and necrotic myocytes following FACS analysis with the Vybrant® Apoptosis Assay Kit. Panel **A**. represents normoxic myocytes and **B**. Myocytes subjected to the hypoxia/reoxygenation protocol.

In order to ascertain that the H/R protocol had been successful, significant differences between the levels of viable, apoptotic and necrotic myocytes were required in comparison with normoxic myocytes which were not subjected to the H/R protocol (as depicted in Figure 2.13).

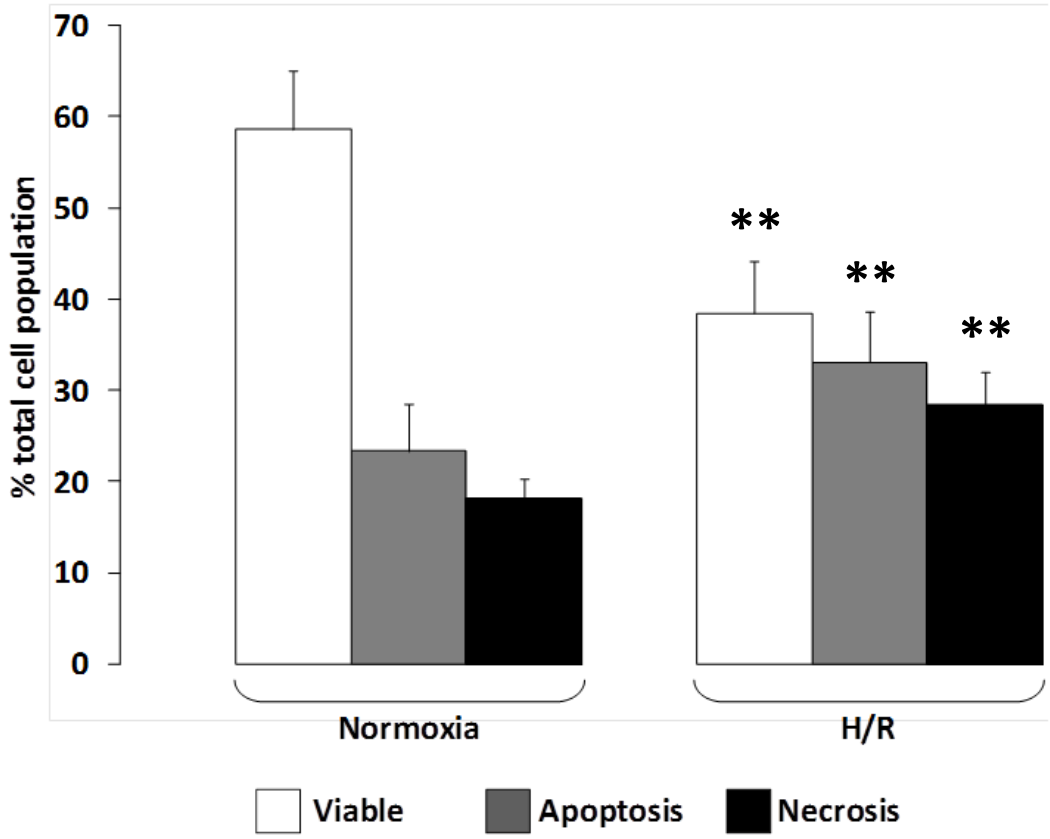


Figure 2.13: The effect of H/R protocol on levels of viable, apoptotic and necrotic cardiac myocytes as determined by the Vybrant® Apoptosis Assay Kit. For all studies conducted using this method, normoxic myocytes were analysed to ensure that the H/R protocol had been successful. **p<0.01 vs. respective normoxic control group. Results represented as mean + SEM, n=10.

2.4.6 Preparation for fluorescence activated cell sorting (FACS) analysis: caspase-3, Akt and BAD assays

The protocol for the intracellular staining of cleaved caspase-3 and phospho-Akt were identical, with the exception of the antibody used. In both cases, the antibody was conjugated to a fluorochrome, which was Alexa Fluor® 488 and Alexa Fluor® 647 fluorescent dyes for cleaved caspase-3 and phospho-Akt (Ser_{473}) protocols respectively. As phospho-BAD (Ser_{112}) primary antibody was not conjugated to a fluorochrome, the protocol was modified to allow for incubation with a secondary, fluorescent antibody prior to flow cytometric analysis. All antibodies were specific solely for the activated form of the proteins, being the cleaved caspase-3 protein and phosphorylated Akt (Ser_{473}) and BAD (Ser_{112}) proteins. The proteins were of particular interest as cleaved caspase-3 and BAD are commonly involved in the activation of apoptotic pathways and Akt is commonly activated by phosphorylation to inhibit apoptotic processes, and is a component of the reperfusion injury salvage kinase (RISK) pathway. All three proteins have been previously shown to play an instrumental role in the regulation of myocardial I/R injury.

After the 4 hour re-oxygenation period, the myocytes were transferred from the 24 well plate to labelled 1.5 ml Eppendorf® centrifuge tubes, as in the cell death assay protocol. The cells were centrifuged at 1200 rpm for 2 minutes before the supernatant was aspirated and discarded. The cells were re-suspended in 250 µl phosphate buffered saline (PBS) before 250 µl 6% formaldehyde solution was added (to give final concentration of 3% formaldehyde). The cells were fixed by incubating for 10 minutes

at 37 ^{o}C, followed by 1 minute incubation on ice. The myocytes were then centrifuged at 1200 rpm for 2 minutes, after which the supernatant was removed.

The cell membranes were then permeabilised by re-suspending in ice-cold 90% methanol solution and incubating on ice for 30 minutes. Following permeabilisation, all samples were washed twice with 0.5% BSA diluted in PBS (incubation buffer) at 4 ^{o}C. This involved centrifugation at 1200 rpm for 2 minutes, removal of the supernatant and subsequent re-suspension in incubation buffer all of which were repeated for the second wash. Myocytes were spun at 1200 rpm for 2 minutes and, following aspiration of the supernatant, re-suspended in 100 µl incubation buffer before 10 minutes incubation at room temperature. During this incubation period, the antibody dilutions were prepared to give a final volume of 100 µl per tube with 1:100 dilution of antibody.

Once the incubation period had elapsed, the myocytes were centrifuged at 1200 rpm for 2 minutes. The supernatant was removed and cells re-suspended in 100 µl of the prepared antibody dilution. All tubes containing cells were covered in foil, to eliminate any loss of fluorescence, and incubated for 1 hour at room temperature. At the end of this time the cells were centrifuged at 1200 rpm for 2 minutes and washed once in incubation buffer as previously described.

The cells were re-suspended in 500 µl PBS thereby allowing for immediate FACS analysis using the FL-1 channel as both Alexa Fluor® 488 and Alexa Fluor® 647 are excited and emit on this channel by red light. Cell Quest® software was used to plot histograms for each group (all concentrations of ipratropium, normoxic control and H/R control) showing the mean fluorescence of 10,000 cell counts.

For the studies where levels of phospho-BAD were measured, the primary antibody was not conjugated to a fluorochrome. Therefore, following the final wash in incubation buffer, myocytes were re-suspended in 200 µl of incubation buffer per tube with 1:1000 dilution of secondary Alexa Fluor® 488 antibody for 30 minutes at room temperature in the dark. Cells were centrifuged for 2 minutes at 1200 rpm and washed twice with 200 µl incubation buffer, as before, prior to re-suspension in 500 µl PBS and analysis on the flow cytometer. These protocols allowed quantification of the levels of cleaved caspase-3, phosphorylated-Akt and phosphorylated-BAD, depending on the assay. These protocols were carried out according to the manufacturer's instructions (New England Biolabs, Hitchin, UK).

Figures 2.14, 2.15 and 2.16 show representative scatter plots from the flow cytometer indicating the levels of caspase-3, Akt (Ser_{473}) and BAD (Ser_{112}), respectively, in myocytes under normoxic conditions (panel **A** for all Figures) and following H/R protocol (panel **B**) and following H/R and ipratropium administration at the onset of re-oxygenation (panel **C**).

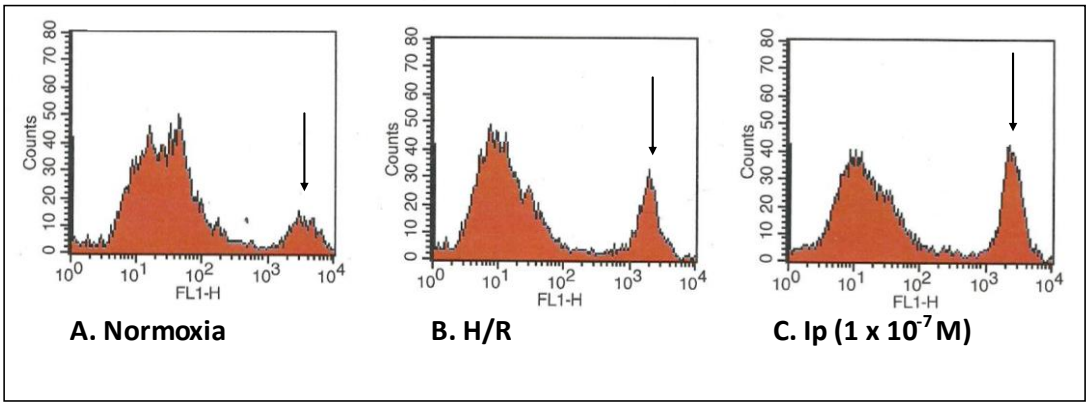


Figure 2.14: Representative flow cytometric scatter plots to show levels of activated cleaved caspase-3 in normoxic cardiac myocytes (**A**), following hypoxia/re-oxygenation protocol (**B**) and with ipratropium administration at the onset of re-oxygenation (**C**). Arrows indicate the peaks which represent cleaved caspase-3 levels.

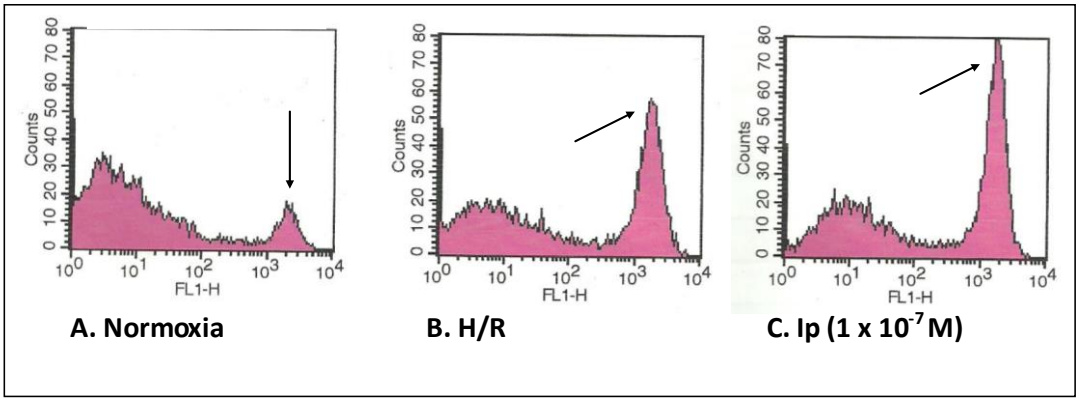


Figure 2.15: Representative flow cytometric scatter plots to show levels of phosphorylated Akt (Ser_{473}) in normoxic cardiac myocytes (**A**), following hypoxia/re-oxygenation protocol (**B**) and with ipratropium administration at the onset of re-oxygenation (**C**). Arrows indicate the peaks which represent phosphorylated Akt levels.

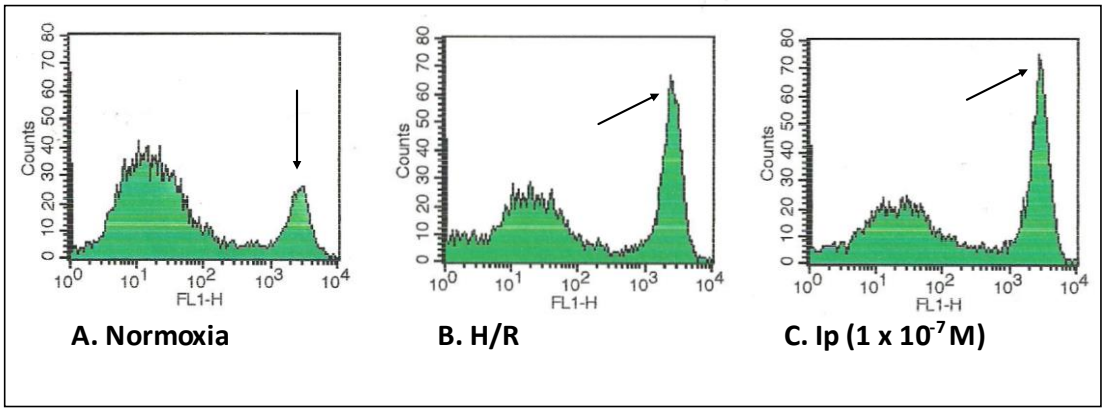


Figure 2.16: Representative flow cytometric scatter plots to show levels of phosphorylated BAD (Ser_{112}) in normoxic cardiac myocytes (**A**), following hypoxia/re-oxygenation protocol (**B**) and with ipratropium administration at the onset of re-oxygenation (**C**). Arrows indicate the peaks which represent phosphorylated BAD levels.

2.5 Myocyte model of oxidative stress

2.5.1 Experimental protocol

Ventricular myocytes were plated onto laminin coated Petri dishes (35 mm diameter) and incubated for ≥2 hours to allow adhesion of the cells. The supernatant, including affluent cells was aspirated and discarded. The adhered myocytes were incubated in microscopy buffer (modified Krebs-Ringer's buffer with 1×10^{-2} M HEPES and 1.2×10^{-6} M $CaCl_2$) containing 3×10^{-6} M, tetramethylrhodamine methyl ester (TMRM) for 15 minutes. Cells were washed with microscopy buffer and then incubated for a further 10 minutes as detailed below. Plates of cells were randomly assigned to one of the drug treatment groups and incubated for a further 15 minutes with 1×10^{-9} M – 1×10^{-7} M ipratropium, 2×10^{-6} M FCCP, 2×10^{-7} M CsA, 1×10^{-7} M ACh or 1×10^{-7} M ipratropium ± CsA or ACh (concentrations as before). To view and analyse myocytes, dishes were placed on the stage of a Zeiss 510 CLSM confocal microscope equipped with 20x objective lens (NA 1.3) and a heated chamber. A 585-nm long pass filter allowed detection of TMRM when viewing the cells. Recording and analysis was facilitated by use of the Zeiss software package, LSM® (version 2.8). Laser stimulation via 543-nm emission line of HeNe laser was used to induce oxidative stress. Prior to laser stimulation, the cationic TMRM selectively localises in the negatively charged inner-membrane of the mitochondrion in a potential-dependent manner. Laser stimulation initiates photodecomposition of TMRM thus generating mitochondrial reactive oxygen species, leading to disruption of the mitochondrial membrane. Depolarisation (Dep) (Figures 2.17 and 2.18) was measured as the time at which the

TMRM started to become evenly distributed throughout the cell and is indicative of loss of mitochondrial membrane potential. Subsequent hypercontracture (Hyp) of myocytes occurs shortly afterwards due to ATP depletion. The time to both Dep and Hyp were recorded.

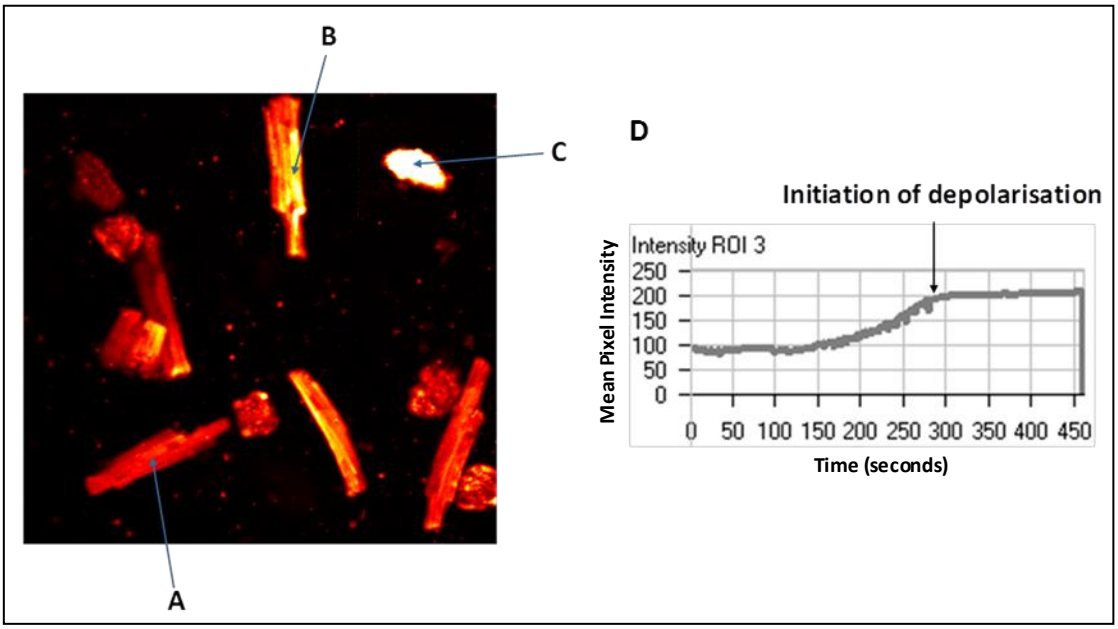


Figure 2.17: A, B and C show TMRM loaded adult rat ventricular myocytes as viewed at x40 magnification under a confocal microscope. A .shows a myocyte prior to depolarisation where TMRM can been seen as isolated, yellow areas within the myocyte, indicative of TMRM localised in the mitochondria. B. shows a myocyte following depolarisation where TMRM passes into the cytosol, as shown by movement of fluorescence across the myocyte. C. Shows a myocyte following hypercontracture due to ATP depletion. Panel D is a representative trace from LSM® (version 2.8), where the intensity of fluorescence was measured following laser stimulation. The increase in intensity, as shown, is indicative of the initiation of depolarisation.

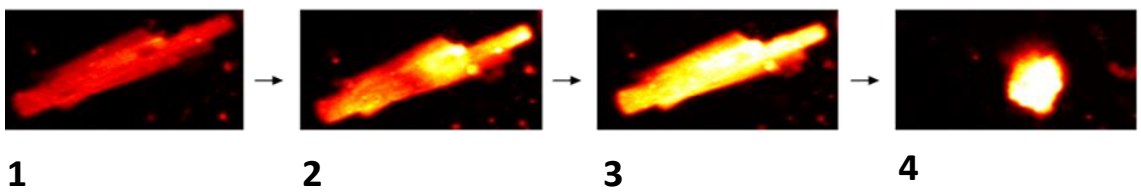


Figure 2.18: Representative TMRM loaded adult rat ventricular myocyte as exposed to oxidative stress following laser stimulation. Panel 1 shows the myocyte prior to depolarisation, 2 and 3 show the onset of and late depolarisation. Panel 4 shows hypercontraction of the myocyte due to ATP depletion.

2.6 Western Blotting

2.6.1 Background to the method

Despite some controversy as to who initially developed the technique, Western blotting is generally attributed to Harry Towbin's laboratory in 1979 (Towbin 2009). The principle builds on that of Southern blotting except instead of separation and identification of DNA, proteins are electrophoretically separated on the basis of their molecular weight. Following electrophoresis, proteins are transferred onto a membrane (generally PVDF) and then the proteins of interest can be identified via specific antibody binding. The levels of specific proteins can then be visualised and identified via computerised densitometry.

2.6.2 Tissue preparation

In order to collect tissue for Western blots, a modified perfused rat heart protocol was used. Briefly, hearts were mounted and perfused with KHB, as before, for a period of 20 mins stabilisation, 35 mins ischaemia and 5, 15 or 120 mins reperfusion (protocol represented in Figure 2.19). As hemodynamic function was not ascertained, atriotomy did not occur. Ischaemia and drug administration were conducted as previously described, and the following experimental groups were used: normoxic, I/R control, 1 $x10^{-9}$ M – 1 x 10^{-7} M ipratropium, 2 x 10^{-7} M CsA, 1 x 10^{-7} M ACh or 1 x 10^{-7} M ipratropium ± CsA or ACh (concentrations as before). Following reperfusion, hearts were dissected to remove the risk area of the left ventricle. The tissue was then

immediately wrapped in foil and placed in liquid nitrogen until frozen. All tissue for Western blotting analysis was then stored at -80 ^{o}C before preparation for experimental use.

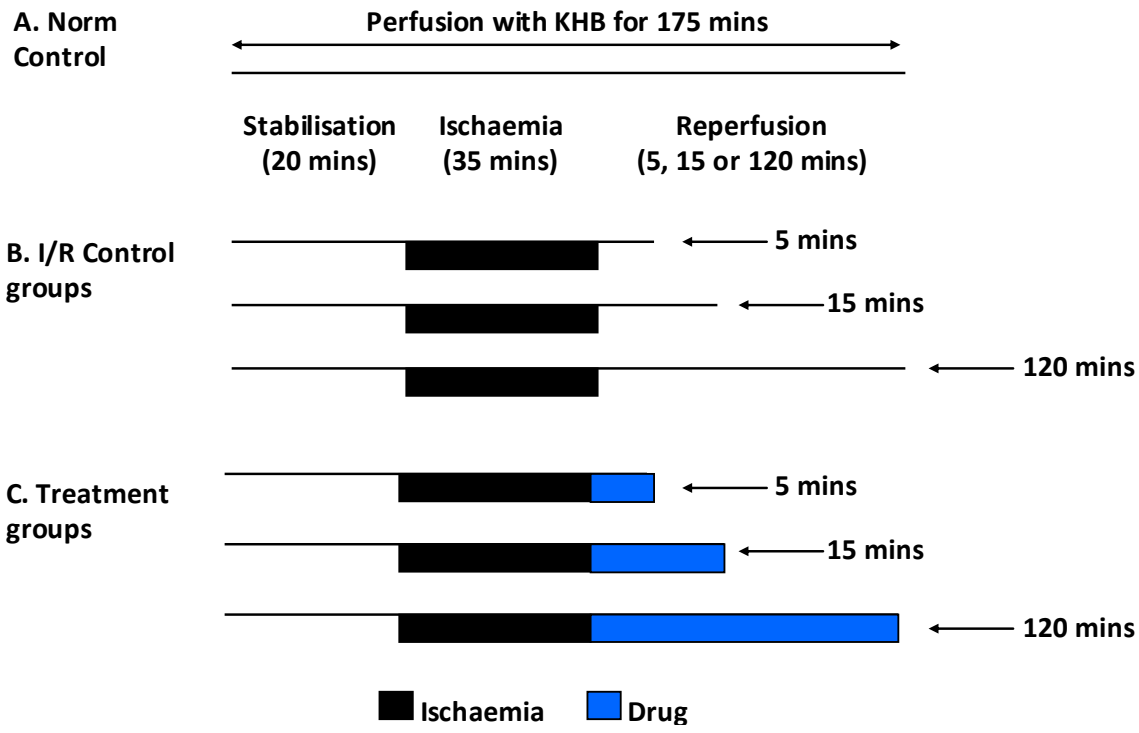


Figure 2.19: Experimental protocol to detail time points at which tissue was collected for Western blot analysis. For all groups subjected to drug administration, all drugs were administered at the onset of reperfusion and hearts were perfused with KHB containing the drugs for the entire reperfusion period.

2.6.2 Tissue homogenisation, protein concentration estimation and storage

Tissue was homogenised with use of an IKA Labortechnik T25 homogeniser in 400 µl lysis buffer (1 x 10^{-1} M NaCl, 1 x 10^{-2} M Tris Base (pH 7.6), 1 x 10^{-3} M EDTA (pH 8.0), 2 x 10^{-3} M Sodium pyrophosphate, 2 x10^{-3} M NaF, 2 x 10^{-3} M β-glycerophosphate, 0.1 mg.ml^{-1} PMSF, 0.1 µg.ml^{-1} aprotinin and leupeptin). The homogenate was placed into

labelled 1.5 ml centrifuge tubes and centrifuged at 11,000 rpm for 10 minutes (at 4 ^{o}C). The supernatant, containing protein, was collected and transferred into clean 1.5 ml centrifuge tubes. The pelleted homogenate was discarded.

Protein concentration was ascertained via NanoDrop spectrophotometric analysis at 280 nm with use of a NanoDrop 1000 Spectrophotometer (NanoDrop Technology, Delaware, USA). 100 µl of each sample was then diluted in 100 µl of sample buffer (2.5 x 10^{-1} M Tris-HCl (pH 6.8), 10% Glycerol, 0.006% Bromophenol blue, 4% SDS, β-mercaptoethanol (pH 6.8)) and subsequently incubated at 95 ^{o}C for 10 minutes. All samples were stored at -20 ^{o}C before defrosting for experimental use.

2.6.3 Electrophoresis and blotting protocol

Dilution in sample buffer (2.5 x 10^{-1} M Tris-HCl (pH 6.8), 10% Glycerol, 0.006% Bromophenol blue, 4% SDS, β-mercaptoethanol (pH 6.8)) occurred to provide 60 µg protein for each sample to be separated via electrophoresis using precast gels and Mini PROTEAN II system (both Bio-Rad, Hemel Hempstead, UK). Gels were placed in the electrode assembly unit and the inner chamber was filled with approximately 125 ml running buffer (14.42 g.l^{-1} glycine, 1.0 g.l^{-1} SDS, 3.0 g.l^{-1} Tris Base). The plastic combs were removed from the gels and wells were loaded with 15 µl of sample buffer and protein sample, which contained 60 µg protein. Molecular weight markers were used in at least one well per gel (New England Biolabs, Ipswich, MA, USA or Bio-Rad, Hemel Hempstead, UK). Electrophoresis took place for 90 minutes at 130 V, with use of a

Power-Pac 3000 (Bio-Rad, Hemel Hempstead, UK) (representative image showing electrophoresis in Figure 2.20).

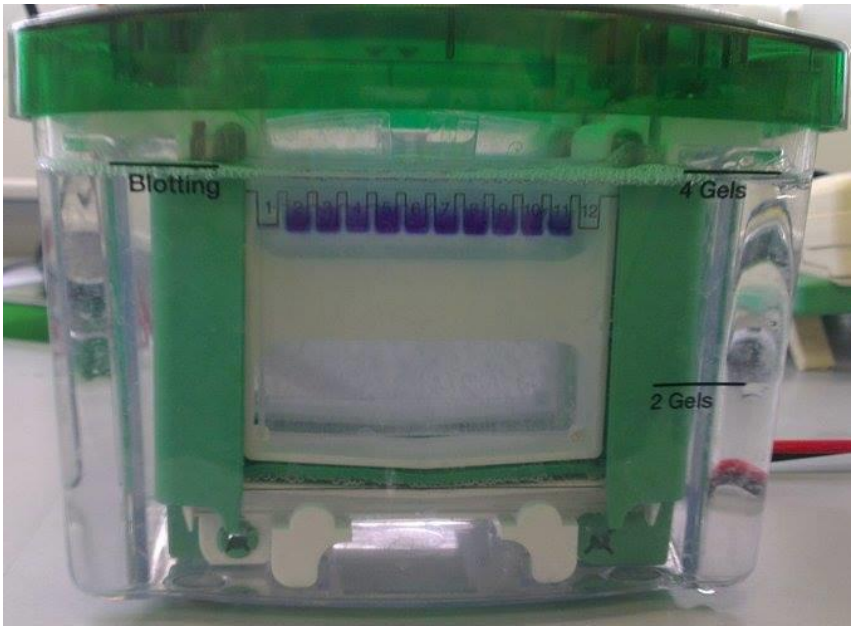


Figure 2.20: Photograph to show gel electrophoretic separation of homogenised adult rat ventricular tissue in sample buffer. As denoted by the pre-cast gels, wells were labelled 1-12 and the movement of the sample can be seen as the blue dye (sample buffer, containing protein) moving from the bottom of the wells.

Transfer to the membrane occurred via use of BioRad TransBlot Turbo system prior to blocking (TBST + 5% milk) and incubation with primary antibody, phospho-Akt (Ser_{473}), phospho-Erk1/2 (Thr_{202}/Thr_{204}), phospho-SAPK/JNK (Thr_{183}/Tyr_{185}), overnight, at 4 ^{o}C. Following 3 x 5 minute washes with TBST, all membranes were incubated with anti-rabbit IgG, HRP-linked antibody on an orbital shaker for 2 hours, at room temperature. After 3 x 5 minute washes with TBST, membranes were subsequently probed and analysed using SuperSignal West Femto® (Thermo) and Quantity One® (Bio-Rad) software for densitometric analysis. Membranes were stripped in boiling water for five

minutes, prior to incubation overnight, with primary Akt, Erk1/2 or JNK in order to ascertain levels of total protein in the samples (analysed as above). Following densitometric analysis for total protein, all membranes were stripped and probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (visualised and analysed as before) in order to assess equal loading. For all three proteins assessed and at all time points studied, it was necessary to identify significant differences between normoxic and I/R treatment groups to show that the experimental protocol had been successful (representative plots are shown in Figure 2.21).

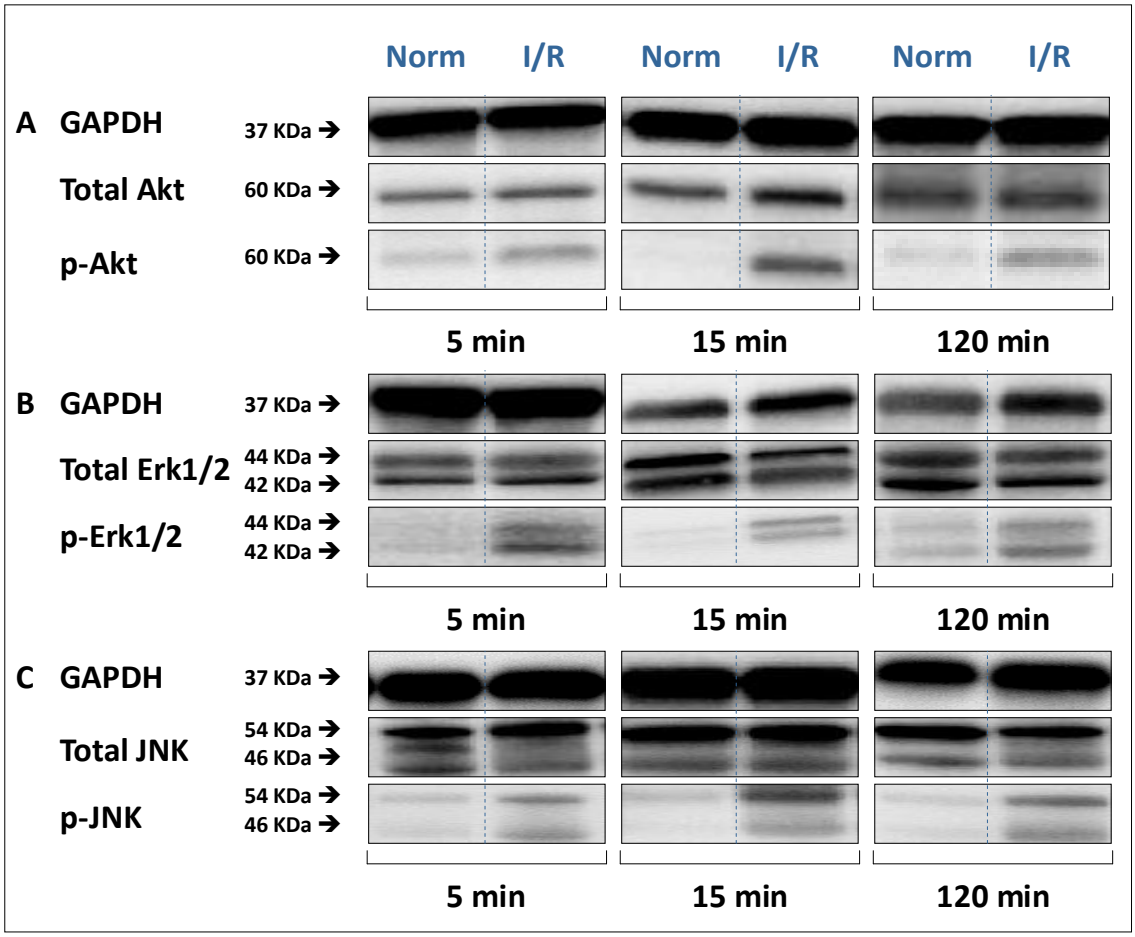


Figure 2.21: Representative Western blots to show the differences between basal (normoxic, norm) levels of (**A**) phospho-Akt, (**B**) phospho Erk1/2 and (**C**) phospho-JNK, and following 20 minutes stabilisation, 35 minutes regional ischaemia and 5, 15 and 120 minutes reperfusion.

2.7 Statistical Analysis

For all data, results were presented as group means + standard error of the mean (SEM) and statistical significance was ascertained using SPSS statistical software (IBM® SPSS® Statistics, version 20).

For all data, SPSS was employed to analyse data via use of a one-way ANOVA (analysis of variance) and Fisher's LSD (least significant difference) post hoc test. A p value of ≤0.05 was considered as being statistically significant.

The only exception to this was that for all haemodynamic parameters (LVDP, HR and CF) were expressed as a percentage of the mean value for stabilisation (first 20 minutes). Data were analysed using a two way ANOVA, also with Fisher's LSD post hoc. A p value of ≤0.05 was considered as being statistically significant.

2.8 Exclusion Criteria

For the Langendorff studies, after the initial 20 minutes stabilisation period, hearts were excluded from further experimental protocol if they exhibited one of more of the following: Average LVDP of less than 100 mmHg, average heart rate of less than 250 bpm, average coronary flow of >20 $ml.min^{-1}$ or obvious, persistent arrhythmias (as determined via visual inspection of the heart and LabChart trace).

Successful ischaemia was established by significant decrease (minimum 30%) in the LVDP and coronary flow as well as myocardial blanching at the site of the snare. Following analysis of haemodynamic function, hearts which did not comply with these were excluded from the study.

Following the myocyte isolation protocol, myocyte isolations producing less than 65% viability were disregarded. In addition, myocyte isolations not exhibiting clear striations were also excluded from the study. Any myocytes from isolations where myocyte plasma membrane blebbing was apparent were considered as apoptotic, and therefore non-viable, and also excluded. Following H/R, a 40% drop in cell viability was necessary to warrant inclusion of the myocytes in the work.

Chapter 3 Ipratropium exacerbates myocardial injury following simulated ischaemia/reperfusion *in vitro*

3.1 Chapter introduction and purpose

Within the past decade, there has been much controversy as to whether anti-cholinergic drugs are associated with increased risk of adverse cardiovascular outcomes in COPD patients, especially those with underlying IHD (Anthonisen et al. 2002, Guite, Dundas and Burney 1999, Lee et al. 2008, Macie et al. 2008, Ringbaek and Viskum 2003, Singh, Loke and Furberg 2008, Wedzicha et al. 2008). In particular, a meta-analysis conducted by Singh *et al*.,in 2008, indicated a 52% increase in risk of mortality due to greater severity and occurrence of cardiovascular events, including MI, due to long-term bronchodilator use, in particular anti-cholinergics (Singh, Loke and Furberg 2008). In addition to this, the majority of COPD patients die from cardiovascular disease with a purported 22% due to complications following an ischaemic event (Lofdahl et al. 2007). COPD is emerging as a disease with multiple causes and co-morbidities, the most common of which is underlying IHD, attributed to underlying, systemic inflammation (Macnee, Maclay and McAllister 2008). However, despite this, to our knowledge, there have been no non-clinical studies to assess the effects of anti-cholinergic compounds, such as ipratropium, in the setting of IHD or MI.

Ipratropium is a non-selective muscarinic antagonist which has been prescribed for the treatment of COPD and, more recently, as an adjunct therapy for asthma for over 30 years (Bathoorn et al. 2008). Within the pulmonary system, the antagonist action of

ipratropium (primarily at the M_3 muscarinic receptor subtype) elicits bronchodilation via inhibition of acetylcholine induced smooth muscle contraction (Bathoorn et al. 2008), as described in detail in Chapter 1, Section 1.9.6. This is therapeutically beneficial as it results in facilitated airflow, thereby ameliorating symptoms of COPD exacerbations (Restrepo 2007).

Clinically, ipratropium is administered by inhalation either through nebulisation or inhaler. Although an extremely effective drug delivery mechanism, inhalers are one of the most frequently abused pharmacological interventions as patients frequently administer doses which are higher than prescribed, and also, it is postulated that up to 90% of the drug may be swallowed rather than inhaled. The bioavailability of ipratropium is considered to be approximately 6.9% (Montuschi et al. 2013). In combination, these factors therefore give rise to the potential of ipratropium to antagonise muscarinic receptors outside the respiratory system and, of particular focus in this study, in the cardiovascular system.

Within the cardiovascular system, endogenous ACh agonise mAChRs (specifically M_1, M_2 and M_3 subtypes), and has been shown to couple to cytoprotective downstream signalling (Borroto-Escuela et al. 2010, Li et al. 2011, Wang et al. 2012).

It is postulated that ipratropium is able to displace ACh at cardiac mAChRs, therefore inhibiting protective downstream pathways. Endogenous muscarinic activation by ACh has also revealed cytoprotective properties against various cellular insults, including conditions of myocardial I/R injury (Budd et al. 2003, Buys et al. 2003, Critz, Cohen and Downey 2005, De Sarno et al. 2003, Krieg et al. 2002, Li et al. 2011, Resende and Adhikari 2009). This includes mechanisms which protect against hypoxia induced

apoptosis (Kim et al. 2008a, Liu et al. 2011a) by regulation of Bcl-2 family proteins and caspase-3, a pivotal caspase in the machinery leading to apoptotic death. In addition to this, activation of the pro-survival kinases Akt and Erk1/2, following ACh induced muscarinic activation, have been shown as cardioprotective via mechanisms which limit apoptosis and inhibit mPTP opening during reperfusion (Sun et al. 2010a, Zang, Sun and Yu 2007).

Further to this, Shaik *et al* have demonstrated that ipratropium is capable of eliciting suicidal erythrocyte death (eryptosis) via a mechanism due to stimulation of increased cytosolic Ca^{2+} activity (Shaik et al. 2012). Despite differences between eryptosis and apoptosis, as apoptosis only occurs in nucleated cells, both of these processes can be initiated by increases in Ca^{2+} concentration, indicating that ipratropium may have the ability to also trigger apoptosis.

Tiotropium bromide (tiotropium, a specific M_3 antagonist also used for the treatment of COPD) has revealed effects which limit acetylcholine mediated fibroblast proliferation (Pieper et al., 2007). This has been further characterised as involving MAPK pathways (particularly Erk1/2 and JNK) (Asano et al. 2010). Although this is clinically beneficial in the context of COPD patients as it limits pulmonary fibrosis and scarring (Pera et al. 2011), it also indicates that anti-cholinergics are capable of eliciting anti-proliferative properties by the blockade of normal mAChR signalling.

It has also been suggested that peripheral blood T-lymphocyte (in particular $CD8^+$) directed apoptosis is increased due to administration of tiotropium bromide and Hemicholinium-3 (a compound which limits the re-uptake of choline, thus limiting acetylcholine synthesis) (Profita et al. 2011). As aforementioned, this is a clinically

beneficial trait, and provides potentially putative pharmacological targets for emerging COPD treatments to limit inflammation. However, taken in combination, in the context of I/R, it could be possible that the limitation of cellular proliferation via anti-cholinergic administration may abrogate the endogenous protective mechanisms of acetylcholine, thereby promoting a shift from muscarinic-driven cytoprotective mechanisms towards pathways which may promote myocyte death. Previous work has also shown that antagonists of the M_3 muscarinic subtypes are pro-apoptotic as well as, in the context of cancer, ACh secreting tumours have been shown to stimulate tumour growth, which is limited by the addition of a muscarinic antagonist (Rosso, 2014).

In this study, it was proposed that the antagonist action of ipratropium may prevent the protective mechanism of acetylcholine and induce myocardial injury.

The purpose of this study was to determine whether ipratropium was capable of eliciting a toxic effect under normoxic conditions and, also, the effect of ipratropium bromide administration in the context of simulated I/R in isolated perfused rat heart (1×10^{-9} M – 1×10^{-6} M) and primary rat ventricular myocyte models ($1 \times 1\ 0^{-11}$ M – 1×10^{-4} M). Acetylcholine (1×10^{-7} M) was also administered concomitantly with ipratropium to ascertain whether the observed effects of ipratropium were due to a specific action on the muscarinic receptors. The involvement of apoptosis, necrosis and cleaved caspase-3 were evaluated by flow cytometry. Additional experiments were undertaken via the use of Z-DEVD-FMK (DEVD, 7×10^{-8} M), a potent caspase-3 inhibitor, to confirm whether the observed effects of ipratropium were dependent on caspase-3 activation. Further to this, Atropine (1×10^{-7} M), another non-selective muscarinic antagonist was

used to support the hypothesis that it was the action of ipratropium at the muscarinic receptors which was responsible for the observed effects.

3.2 Methods

Adult, male, Sprague-Dawley rats (300 g ± 50 g body weight) were used throughout the experiments detailed in this chapter. All experiments were subject to the exclusion criteria detailed in Chapter 2, Section 2.8. For all data, results were presented a group means + standard error of the mean (SEM). For the flow cytometry data, values were normalised to the respective untreated control, which was represented as 100%. Statistical differences were ascertained with the use of one-way ANOVA, with the exception of haemodynamics which where a two-way ANOVA was employed, and Fisher's LSD post-hoc test.

3.2.1 Isolated perfused rat heart model

Following sacrifice by cervical dislocation and excision, rat hearts were mounted on Langendorff perfusion apparatus. For the normoxic studies, hearts were subjected to retrograde perfusion for a total of 175 minutes, ipratropium (1×10^{-8} M – 1×10^{-6} M) was administered following 55 minutes stabilisation, for the remaining 120 minutes of the experiment. This protocol was used in order to time-match Langendorff experiments where the I/R protocol was used. For the experiments using the Langendorff model of I/R (further detail in Section2.3.4), ipratropium (1×10^{-7} M) was added at different time points (0 mins, 20 mins, 40 mins and 55 mins) following the start of the experiment. In subsequent experiments, ipratropium (1×10^{-9} M – 1×10^{-6} M), acetylcholine (1×10^{-7} M), atropine (1×10^{-7} M) and DEVD (7×10^{-8} M) as well as

ipratropium (1 x 10^{-7} M) + acetylcholine (1 x 10^{-7} M), atropine (1 x 10^{-7} M) + acetylcholine (1 x 10^{-7} M) and ipratropium (1 x 10^{-7} M) + DEVD (7 x 10^{-8} M) were administered after 55 mins, the onset of reperfusion. At the end of the experimental protocol, infarct size to risk ratio (I/R %) was determined via Evans blue and TTC staining. Throughout all experiments, left ventricular developed pressure (LVDP, mmHg), heart rate (HR, beats.min^{-1}) and coronary flow (CF, ml.min^{-1}) were measured to assess the stability of the hearts.

3.2.2 Analysis of adult rat ventricular myocytes via flow cytometry

Throughout these studies, simulated I/R was achieved in isolated ventricular rat myocytes using a hypoxia/re-oxygenation protocol (as described in further detail in Section 2.4.1). For the normoxic study, isolated cardiac myocytes were incubated with ipratropium (1 x 10^{-11} M – 1 x 10^{-6} M) for four hours prior to flow cytometric analysis for apoptosis, necrosis and viable myocytes. For the hypoxia/re-oxygenation (H/R) studies, ipratropium (1 x 10^{-9} M – 1 x 10^{-6} M), acetylcholine (1 x 10^{-7} M) and atropine (1 x 10^{-7} M)) as well as ipratropium (1 x 10^{-7} M) + acetylcholine (1 x 10^{-7} M), atropine (1 x 10^{-7} M) + acetylcholine (1 x 10^{-7} M), were administered for four hours, following two hours hypoxia. At the end of the experimental protocol, caspase-3, apoptosis, necrosis and viable myocytes were assessed by flow cytometry.

3.2.3 Analysis of adult rat ventricular myocytes via MTT assay

Isolated adult rat ventricular myocytes underwent two hours hypoxia after which, at the onset of re-oxygenation, ipratropium (1×10^{-9} M – 1×10^{-6} M), acetylcholine (1×10^{-7} M) and atropine (1×10^{-7} M)) as well as ipratropium (1×10^{-7} M) + acetylcholine (1×10^{-7} M), atropine (1×10^{-7} M) + acetylcholine (1×10^{-7} M) were administered for four hours, in the precence of MTT. Myocytes were subsequently lysed and analysed for MTT reductase activity.

3.2.4 Acetylcholine assay

Choline/Acetylcholine Assay Kit (Abcam, UK) was used to determine cell lysate acetylcholine levels in myocytes under normoxic conditions and those subjected to the H/R protocol. The assay was conducted following the manufacturer's instruction.

3.3 Results

3.3.1 Isolated perfused rat heart pilot experiments

The initial study was designed in order to establish whether ipratropium was capable of eliciting myocardial injury under normoxic conditions, in both perfused Langendorff hearts and isolated adult rat ventricular myocytes.

3.3.1.1 The effect of ipratropium on infarct development (I/R %) when administered under normoxic conditions

Isolated perfused rat hearts were subjected to 55 minutes perfusion with Krebs Heinsleit Buffer (KHB) prior to administration of ipratropium (1×10^{-8} M – 1×10^{-6} M) for 120 minutes. Infarct development was ascertained as a percentage of tetrazolium negative tissue compared with tetrazolium positive tissue (Infarct/Risk (%), I/R%). These data demonstrated that in comparison with the untreated normoxic control (5.8 ± 1.7%) there was no statistical increase in I/R% with any of the concentrations of ipratropium tested (8.7 ± 3.5% (1×10^{-8} M), 9.6 ± 2.2% (1×10^{-7} M), 7.2 ± 2.5% (1×10^{-6} M), $p>0.05$, Figure 3.1).

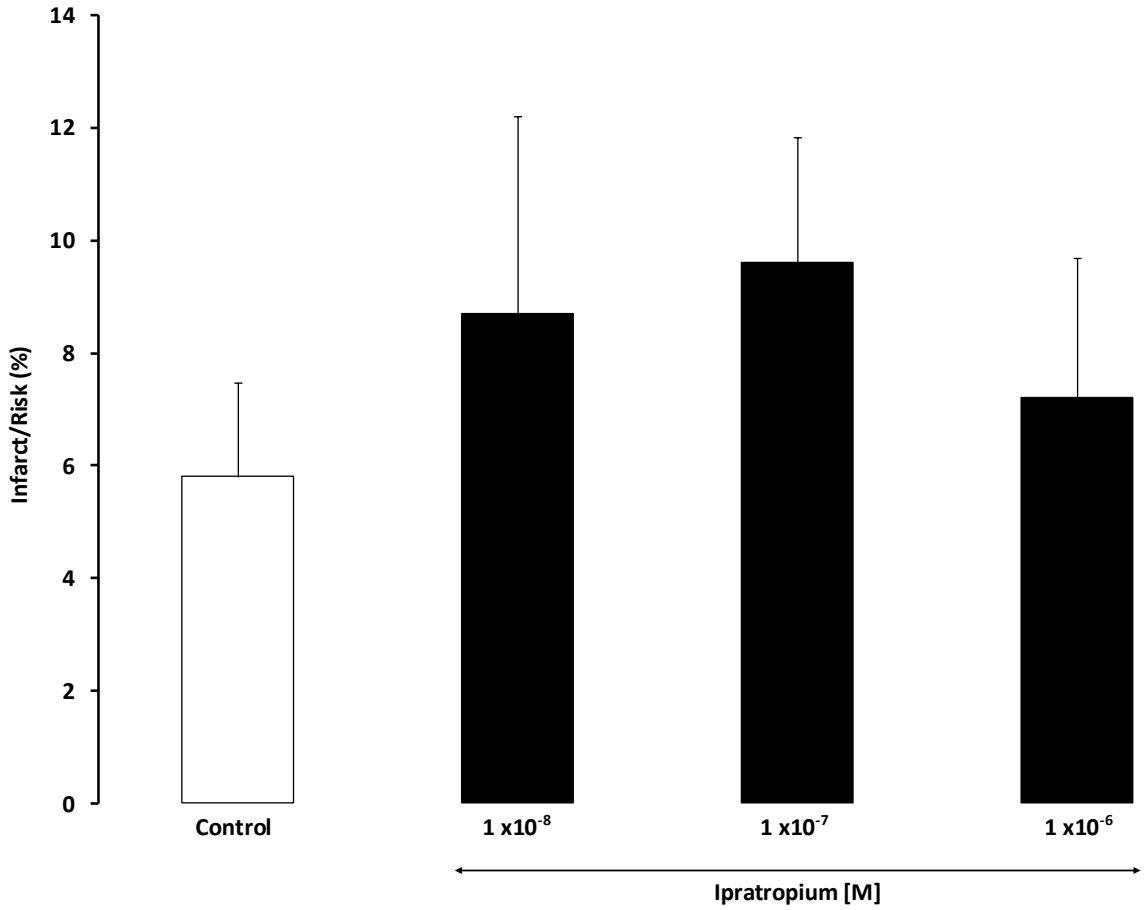


Figure 3.1: Infarct development in normoxic perfused isolated rat hearts. Following 55 minutes perfusion, ipratropium (1 x 10^{-8} M – 1 x 10^{-6} M) was administered for 120 minutes. Results are presented as Infarct size to risk ratio (%). Results expressed as arithmetic mean + SEM, n=5 for all groups, p>0.05.

3.3.1.2 Haemodynamic parameters

Throughout all Langendorff preparation experiments, left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF) were recorded every five minutes for the first 55 minutes and every 15 minutes for the last 120 minutes of the perfusion protocol.

3.3.1.3 Left ventricular developed pressure (LVDP)

Left ventricular developed pressure (LVDP) was ascertained via use of a water filled latex balloon, coupled to a pressure transducer, which was inserted into the left ventricle. Results are expressed as a percentage of the mean LVDP during the stabilisation period (first 20 minutes). There was no significant difference in LVDP in comparison with the normoxic control with the hearts treated with ipratropium (1 x 10^{-8} M) at any time point. However, at the 75, 90, 105 and 120 minutes time points, both 1 x 10^{-7} M and 1 x 10^{-6} M ipratropium showed a significant increase in LVDP in comparison with the control group ($p<0.05$, Figure 3.2).

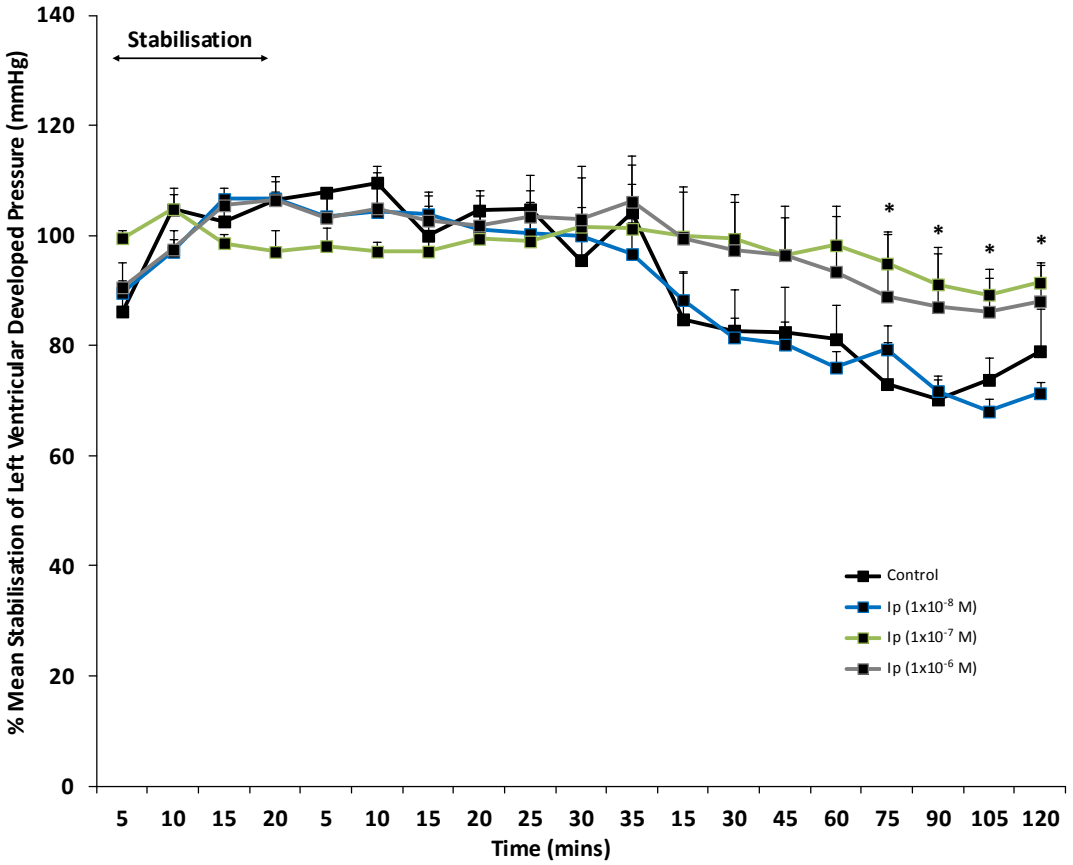


Figure 3.2: Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 55 minutes perfusion with KHB (20 minutes stabilisation) followed by 120 minutes perfusion with KHB ± ipratropium (1 x 10^{-8} M – 1 x 10^{-6} M). *$p<0.05$, Ip (1 x 10^{-7} M and 1 x 10^{-6} M) vs. untreated control. Values expressed as mean percentage of the stabilisation period + SEM, n= 5 for all groups.

3.3.1.4 Heart rate (HR)

Heart rate (HR) was also measured via use of a water filled latex balloon, coupled to a pressure transducer, which was inserted into the left ventricle. Results are expressed as a percentage of the mean HR during the stabilisation period (first 20 minutes). There was no significant difference in HR in comparison with the normoxic control with the hearts treated with ipratropium (1 x 10^{-8} M – 1 x 10^{-6} M) at any time point (Figure 3.3).

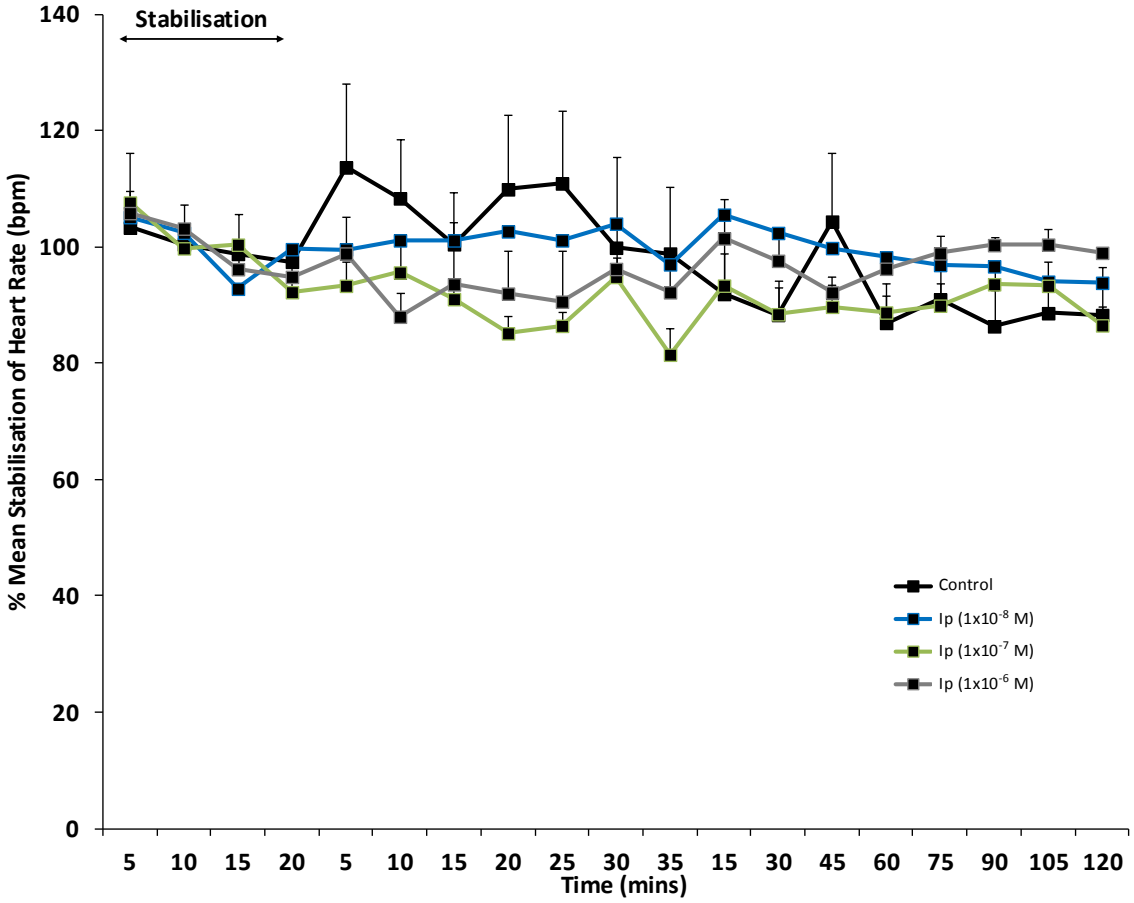


Figure 3.3: Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 55 minutes perfusion with KLB (20 minutes stabilisation) followed by 120 minutes perfusion with KHB ± ipratropium (1 x 10^{-8} M – 1 x 10^{-6} M). Values expressed as mean percentage of the stabilisation period + SEM, n= 5 for all groups $p > 0.05$.

3.3.1.5 Coronary flow (CF)

Coronary flow (CF) measurements were taken by collecting and recording the volume of coronary effluent from perfused hearts for one minute. Results are expressed as a percentage of the mean CF during the stabilisation period (first 20 minutes). There was no significant difference in CF in comparison with the normoxic control with the hearts treated with ipratropium (1 x 10^{-8} M – 1 x 10^{-6} M) at any time point (Figure 3.4).

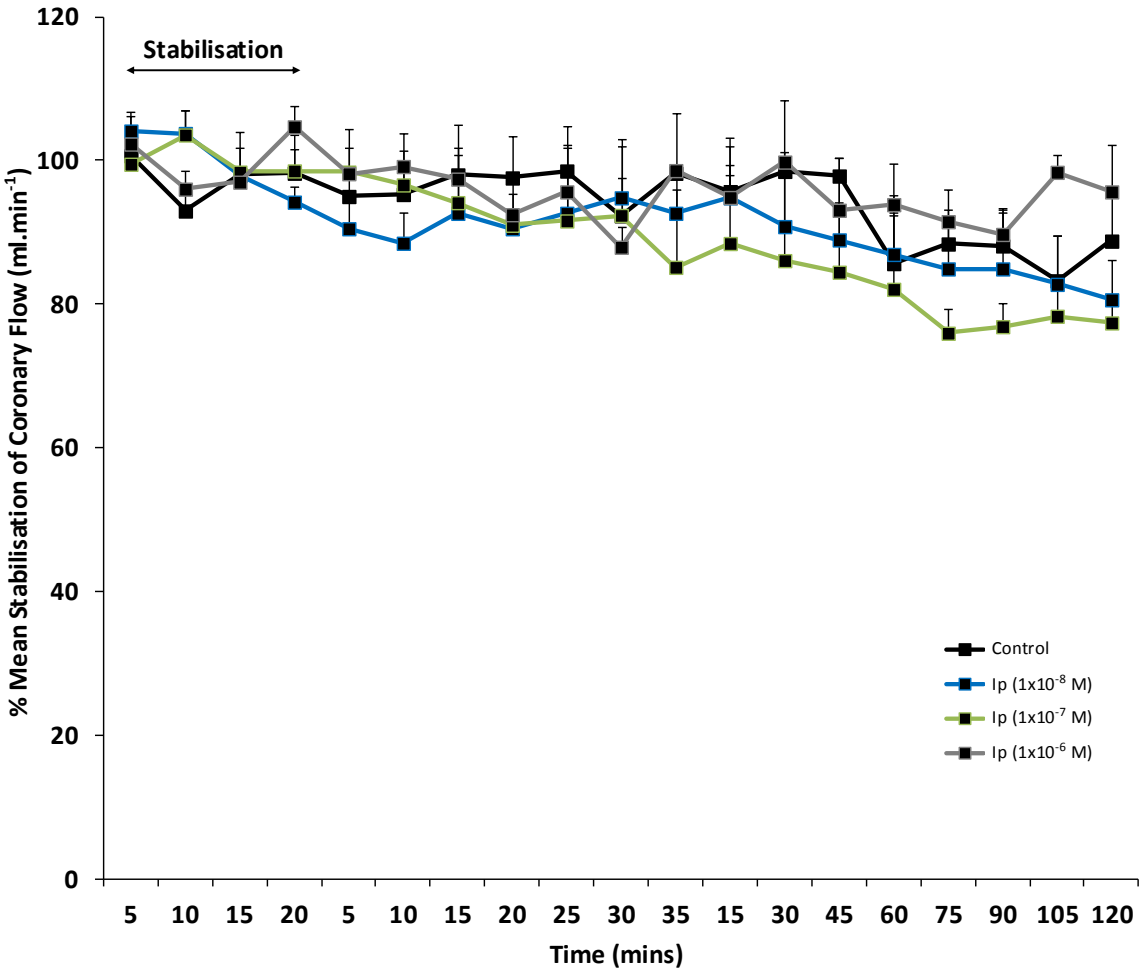


Figure 3.4: Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 55 minutes perfusion with KLB (20 minutes stabilisation) followed by 120 minutes perfusion with KHB ± ipratropium (1 x 10^{-8} M – 1 x 10^{-6} M). Values expressed as mean percentage of the stabilisation period + SEM, n= 5 for all groups, $p > 0.05$.

3.3.2 Assessment of viable, apoptotic and necrotic primary cardiac myocytes under normoxic conditions exposed to ipratropium treatment

This study was carried out to ascertain whether ipratropium (1×10^{-11} M – 1×10^{-6} M) had an effect on cellular viability, apoptosis or necrosis in isolated adult rat ventricular myocytes. Following isolation, normoxic myocytes were exposed to ipratropium treatment for 6 hours and relative cell populations were determined via use of flow cytometry using Vybrant® Apoptosis Assay Kit #10 (Figure 3.5). There was no statistical change in myocyte viability, apoptosis or necrosis in the ipratropium treated groups in comparison with the untreated control at any concentration of ipratropium tested ($p>0.05$).

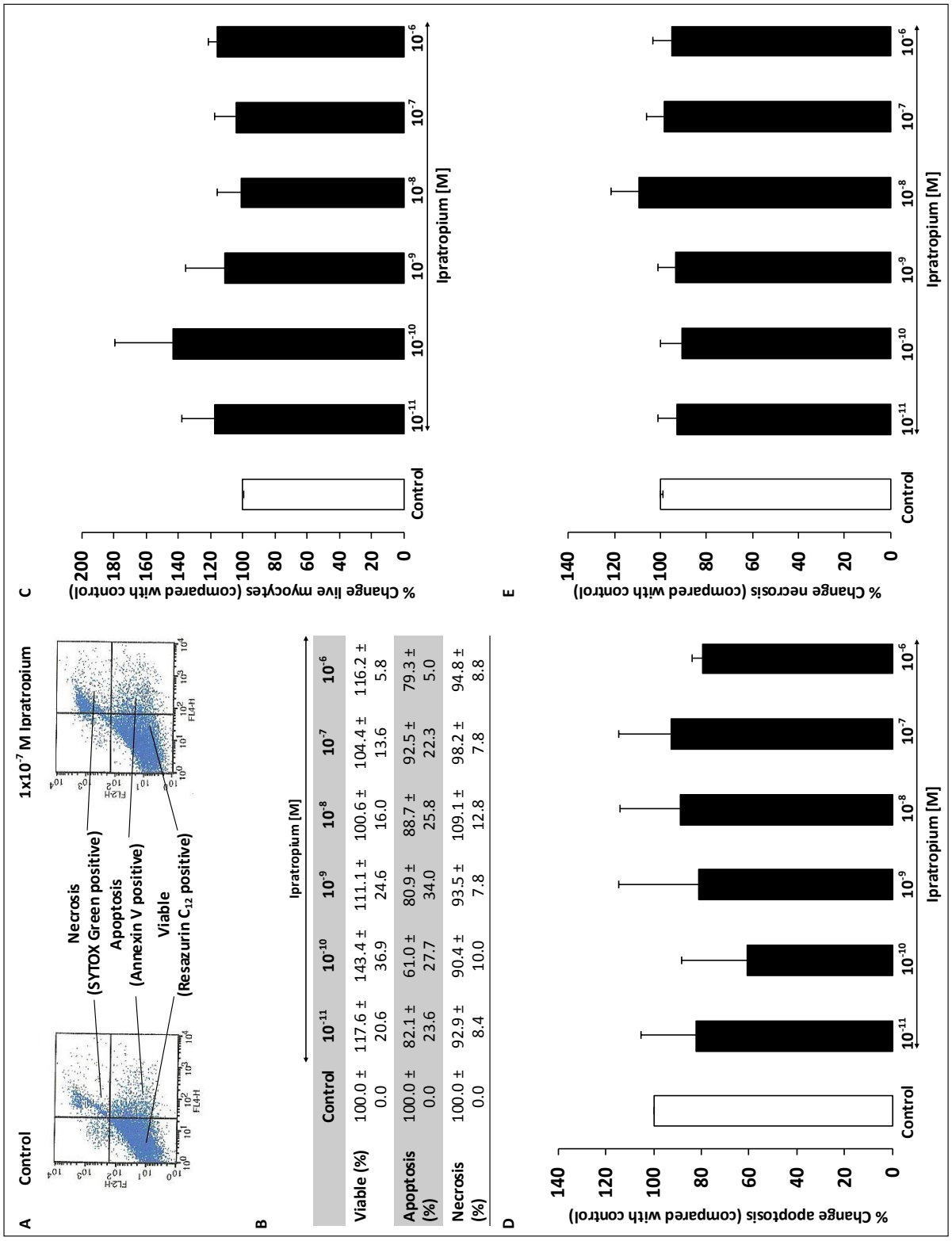


Figure 3.5: Representative flow cytometric scatter graphs (A) showing viable, apoptotic and necrotic cell populations in both untreated and following ipratropium (1 x 10^{-7} M) administration. Assessment of adult rat ventricular myocyte viability (C), apoptosis (D) and necrosis (E) following ipratropium (1 x 10^{-11} M – 1 x 10^{-6} M) treatment under normoxic conditions. Results are represented as mean % change in comparison with the untreated control + SEM (values shown in B) n= 10 for all groups, $p > 0.05$.

3.3.3 Ipratropium administration throughout ischaemia in the absence of reperfusion

Following the elucidation that ipratropium did not significantly increase myocardial injury in isolated perfused rat heart or adult ventricular cardiac myocytes under normoxic conditions, it was determined that ipratropium would be administered during ischaemic conditions in the Langendorff model.

3.3.3.1 The effect of ipratropium on infarct development (I/R %) when administered under ischaemic conditions

Throughout these experiments, both normoxic and ischaemic controls were used. The normoxic control was subjected to 55 minutes perfusion with KHB, whereas the ischaemic control was perfused with KHB for the 20 minutes stabilisation period and 35 minutes regional ischaemia. In the groups treated with ipratropium, ipratropium was added following 20 minutes stabilisation, and throughout 35 minutes ischaemia, after which hearts were removed from the apparatus for Evans blue and TTC staining, prior to analysis (results presented in Figure 3.6). In comparison with the untreated normoxic control (white bar), the untreated ischaemic control (grey bar) showed a statistically significant increase in I/R% (5.8 ± 1.7%, normoxic, vs. 17.1 ± 4.2%, ischaemia, $p<0.01$). In addition to this, at all concentrations of ipratropium tested (1 x 10^{-7} M and 1 x 10^{-6} M), there was a statistical increase in I/R% compared with the normoxic control (5.8 ± 1.7%, normoxic, vs. 18.0 ± 4.5%, Ip, 1 x 10^{-7} M and 17.3 ± 5.0%, Ip, 1 x 10^{-6} M, $p<0.01$). However, in comparison with the untreated ischaemic control

subjected to ischaemia, the ipratropium treated groups were statistically insignificant ($p>0.05$).

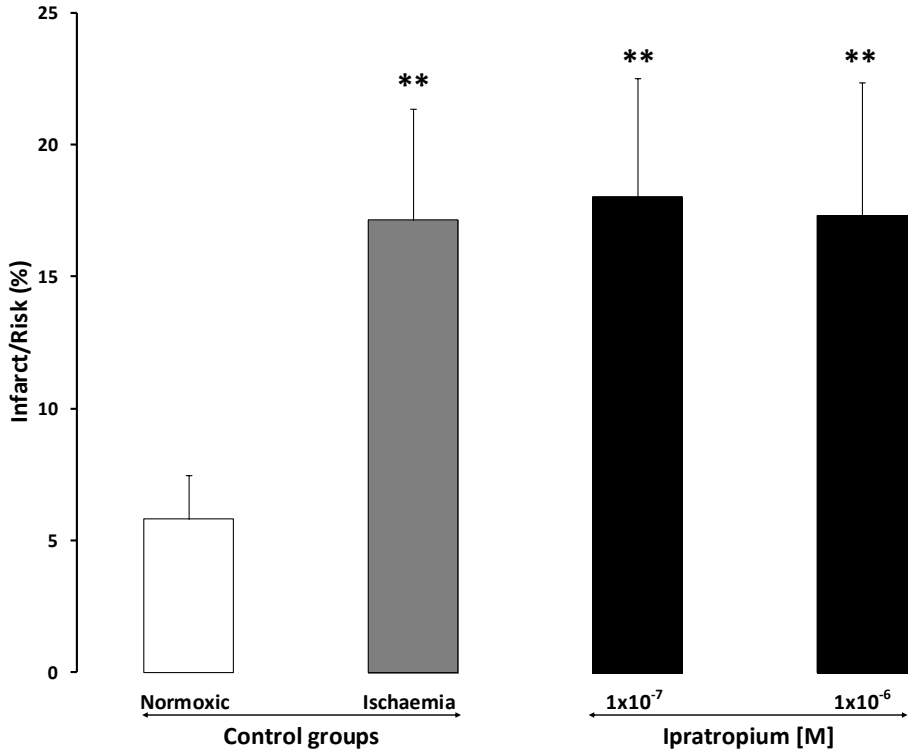


Figure 3.6: Infarct development in the risk zone after administration of ipratropium (1×10^{-7} M and 1×10^{-6} M) at the onset of, and throughout, ischaemia. Results are presented as infarct/risk ratio (Infarct/Risk %). Expressed as arithmetic mean + SEM. **$p<0.01$ vs. normoxic control (white bar), n=5.

3.3.3.2 Haemodynamic parameters

All haemodynamic parameters were measured and expressed as previously described.

3.3.3.3 Left ventricular developed pressure (LVDP)

There was no significant difference in LVDP in comparison with the control group compared with hearts treated with ipratropium (1 x 10^{-7} M or 1 x 10^{-6} M) at any time point ($p>0.05$) (Figure 3.7).

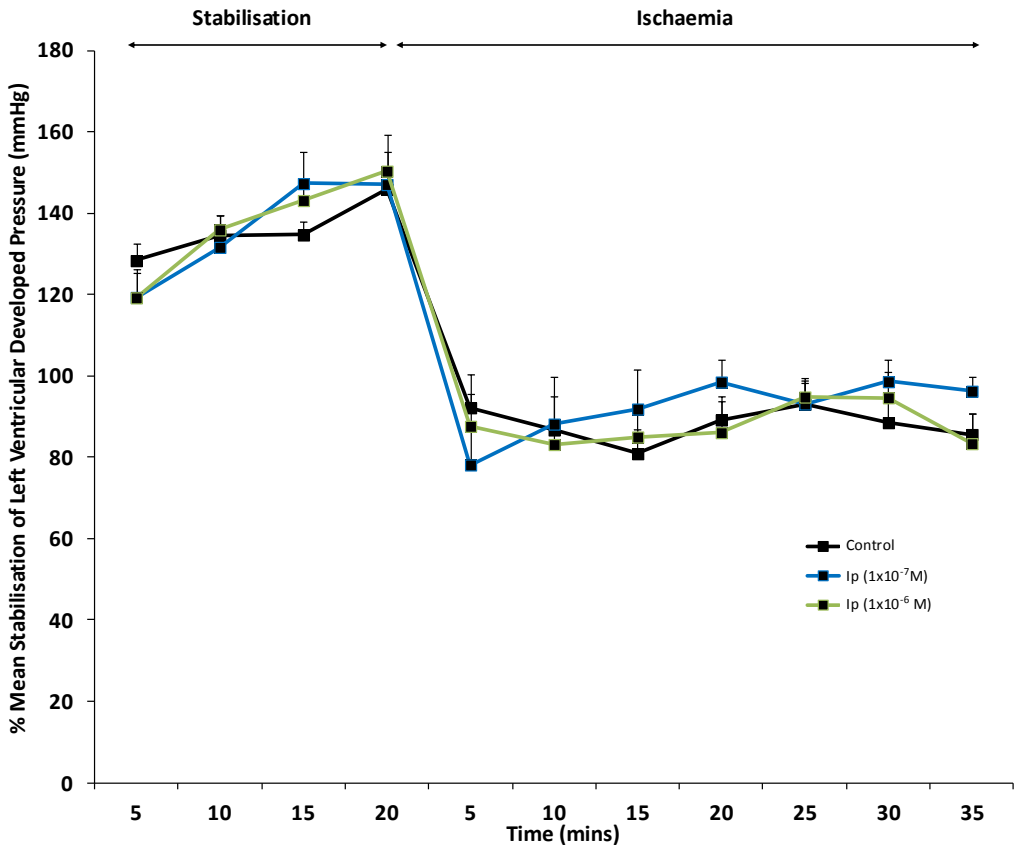


Figure 3.7: Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 20 minutes stabilisation and 35 minutes ischaemia. Ipratropium (1 x 10^{-7} M and 1 x 10^{-6} M) was administered at the onset of, and throughout, ischaemia. Values expressed as mean percentage of the stabilisation period + SEM, n= 5 for all groups, $p > 0.05$.

3.3.3.4 Heart rate (HR)

There was no significant difference in HR in comparison with the control group with hearts treated with ipratropium (1 x 10^{-7} M or 1 x 10^{-6} M) at any time point ($p>0.05$) (Figure 3.8).

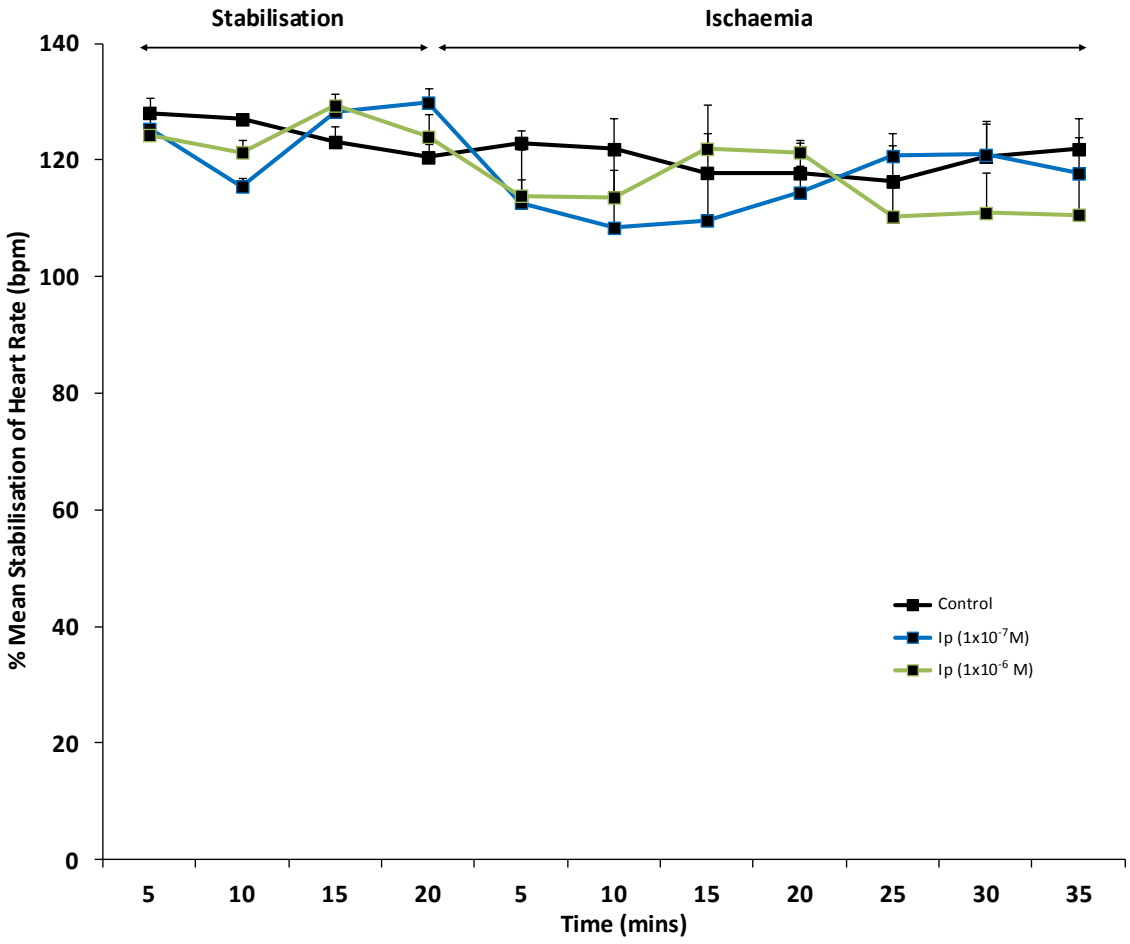


Figure 3.8: Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 20 minutes stabilisation and 35 minutes ischaemia. Ipratropium (1 x 10^{-7} M and 1 x 10^{-6} M) was administered at the onset of, and throughout, ischaemia. Values expressed as mean percentage of the stabilisation period + SEM, n= 5 for all groups, $p > 0.05$.

3.3.3.5 Coronary flow (CF)

There was no significant difference in CF in comparison with the control group with hearts treated with ipratropium (1 x 10^{-7} M or 1 x 10^{-6} M) at any time point ($p>0.05$) (Figure 3.9).

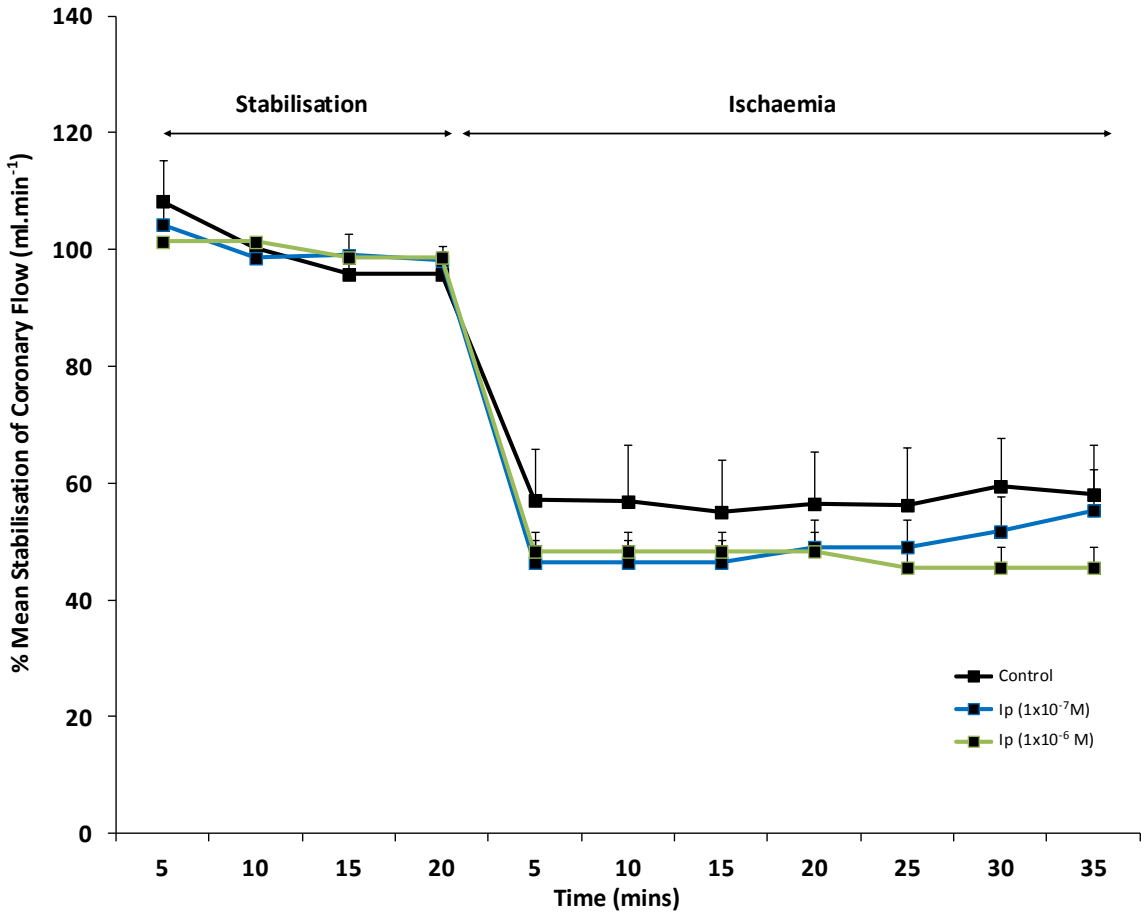


Figure 3.9: Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 20 minutes stabilisation and 35 minutes ischaemia. Ipratropium (1 x 10^{-7} M and 1 x 10^{-6} M) was administered at the onset of, and throughout, ischaemia. Values expressed as mean percentage of the stabilisation period + SEM, n= 5 for all groups, $p > 0.05$.

3.3.4 Ipratropium administration at different time points during ischaemia/reperfusion protocol

In order to elucidate whether ipratropium administration had an effect on the myocardium following ischaemia and reperfusion (I/R), further isolated perfused Langendorff heart studies were conducted whereby hearts were subjected to 20 minutes stabilisation, 35 minutes regional ischaemia (via ligation of the left descending coronary arteries) and 120 minutes reperfusion (as previously described).

3.3.4.1 The effect of ipratropium on infarct development (I/R %) when administered at different time points throughout the ischaemia/reperfusion protocol

As presented in Figure 3.10, ipratropium (1×10^{-7} M) was administered at the onset of stabilisation and throughout the full experimental protocol (0 mins), at the onset of ischaemia (20 mins), 15 minutes before the onset of reperfusion (40 mins) and at the onset of reperfusion (55 mins). In all experimental groups, there was a statistical increase in I/R% in comparison with the untreated control, which was subjected to the same protocol, but perfused with KHB only. Results presented in Table 3.1, $p<0.01$ for all groups in comparison with untreated control.

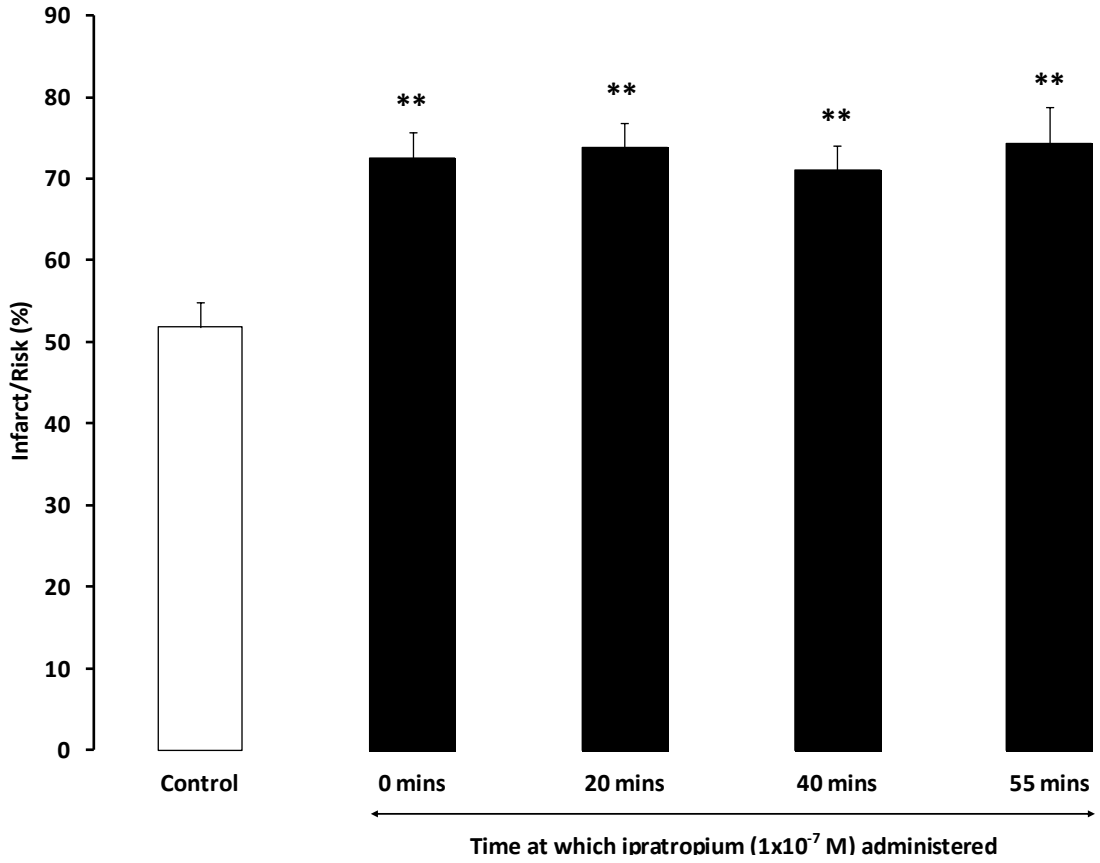


Figure 3.10: Infarct development in the risk zone after administration of ipratropium (1 x 10^{-7} M) at different time points, throughout stabilisation, ischaemia and reperfusion (0 mins), throughout ischaemia and reperfusion (20 mins), administered 15 minutes prior to the end of ischaemia and throughout reperfusion (40 mins) and throughout reperfusion only (55 mins). Results are presented as infarct/risk ratio (Infarct/Risk %). Expressed as arithmetic mean + SEM. **p<0.01 vs. untreated control, n=6.

		Time at which ipratropium (1 x 10^{-7} M) administered			
Group	**Control**	**0 mins**	**20 mins**	**40 mins**	**55 mins**
I/R (%)	51.8 ± 3.0 %	72.6 ± 3.4 % **	73.8 ± 4.7 % **	71.0 ± 3.2 % **	74.3 ± 4.5 % **

Table 3.1: Values for infarct development in the risk zone following ipratropium (1 x 10^{-7} M) administration at different time points stabilisation, ischaemia and reperfusion (0 mins), throughout ischaemia and reperfusion (20 mins), administered 15 minutes prior to the end of ischaemia and throughout reperfusion (40 mins) and throughout reperfusion only (55 mins). Results are presented as infarct/risk ratio (Infarct/Risk %). Expressed as arithmetic mean ± SEM. **p<0.01 vs. control (white bar), n=6.

3.3.4.2 Haemodynamic parameters

All haemodynamic parameters were measured and expressed as previously described.

3.3.4.3 Left ventricular developed pressure (LVDP)

There was no significant difference in LVDP in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups (p>0.05) (Figure 3.11).

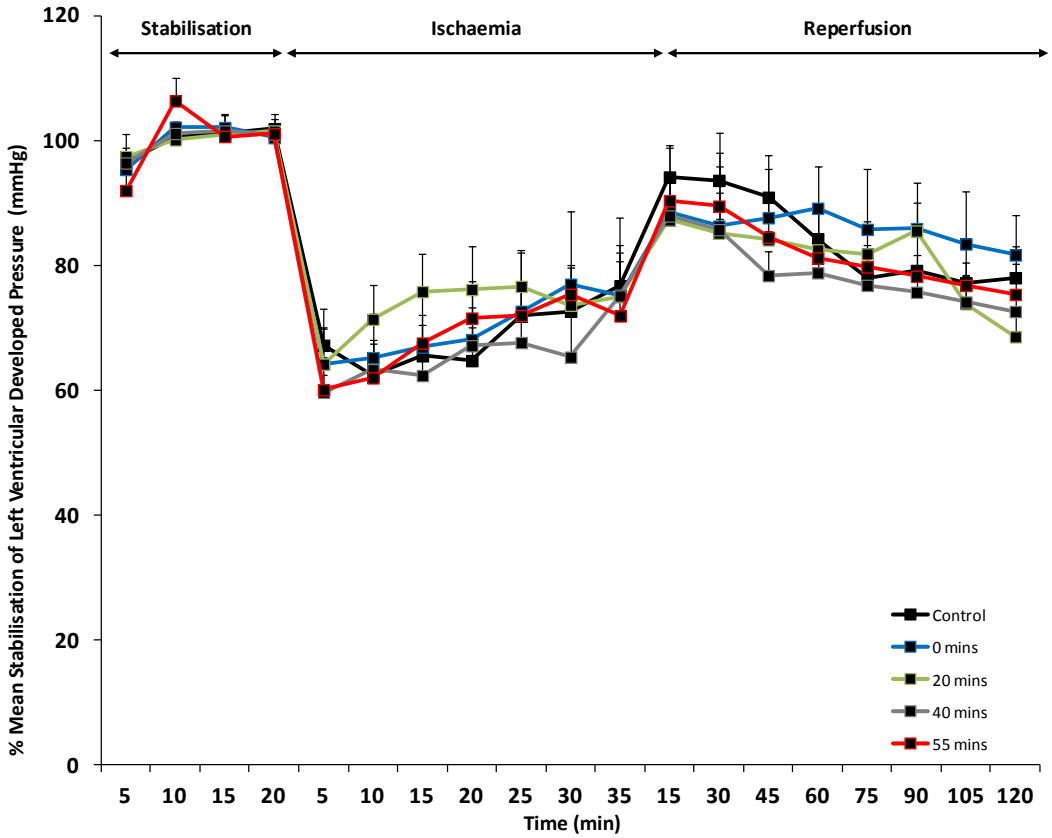


Figure 3.11: Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Administration of ipratropium (1 x 10^{-7} M) occurred at different time points, throughout stabilisation, ischaemia and reperfusion (0 mins), throughout ischaemia and reperfusion (20 mins), administered 15 minutes prior to the end of ischaemia and throughout reperfusion (40 mins) and throughout reperfusion only (55 mins).Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, p > 0.05.

3.3.4.4 Heart rate (HR)

There was no significant difference in HR in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups ($p>0.05$) (Figure 3.12).

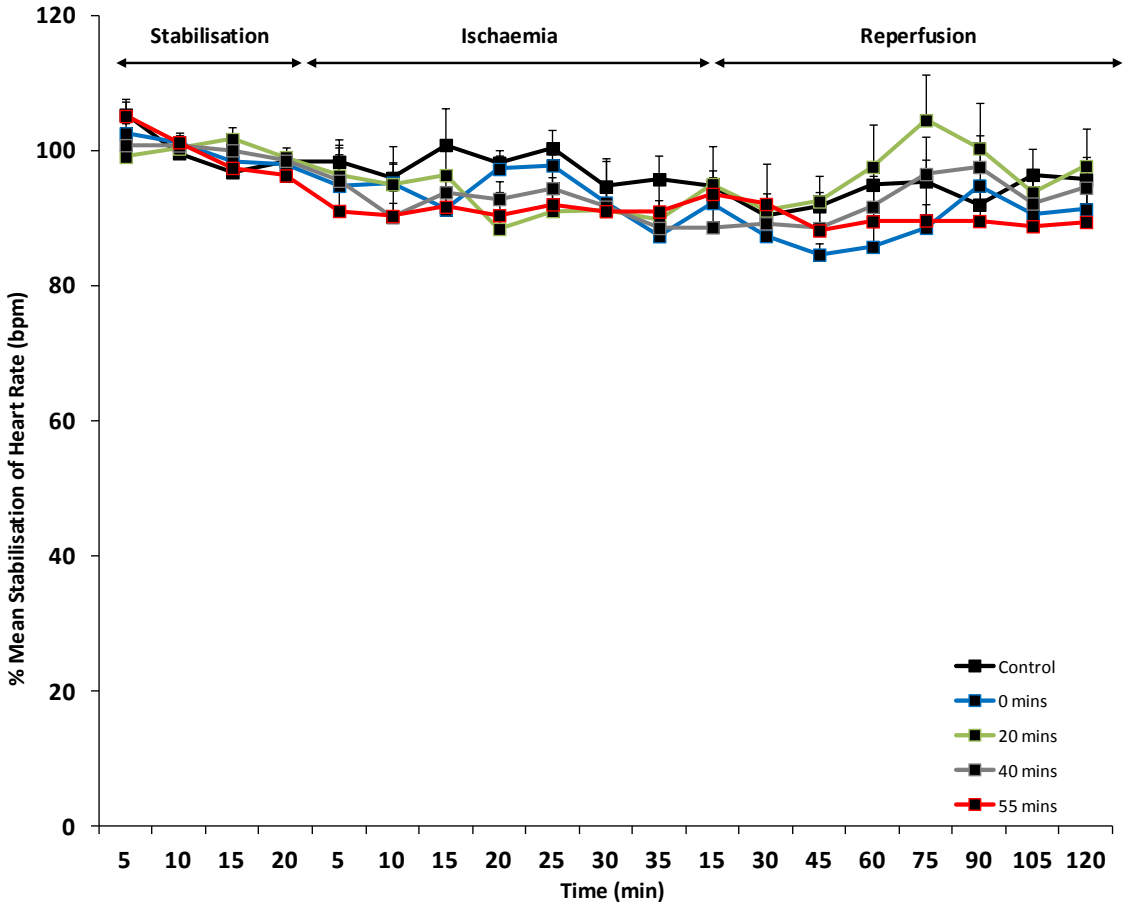


Figure 3.12: Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Administration of ipratropium (1 x 10^{-7} M) occurred at different time points, throughout stabilisation, ischaemia and reperfusion (0 mins), throughout ischaemia and reperfusion (20 mins), administered 15 minutes prior to the end of ischaemia and throughout reperfusion (40 mins) and throughout reperfusion only (55 mins).Values expressed as mean percentage of the stabilisation period ± SEM, n= 6 for all groups, $p > 0.05$.

3.3.4.5 Coronary flow (CF)

There was no significant difference in CF in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups ($p>0.05$) (Figure 3.13).

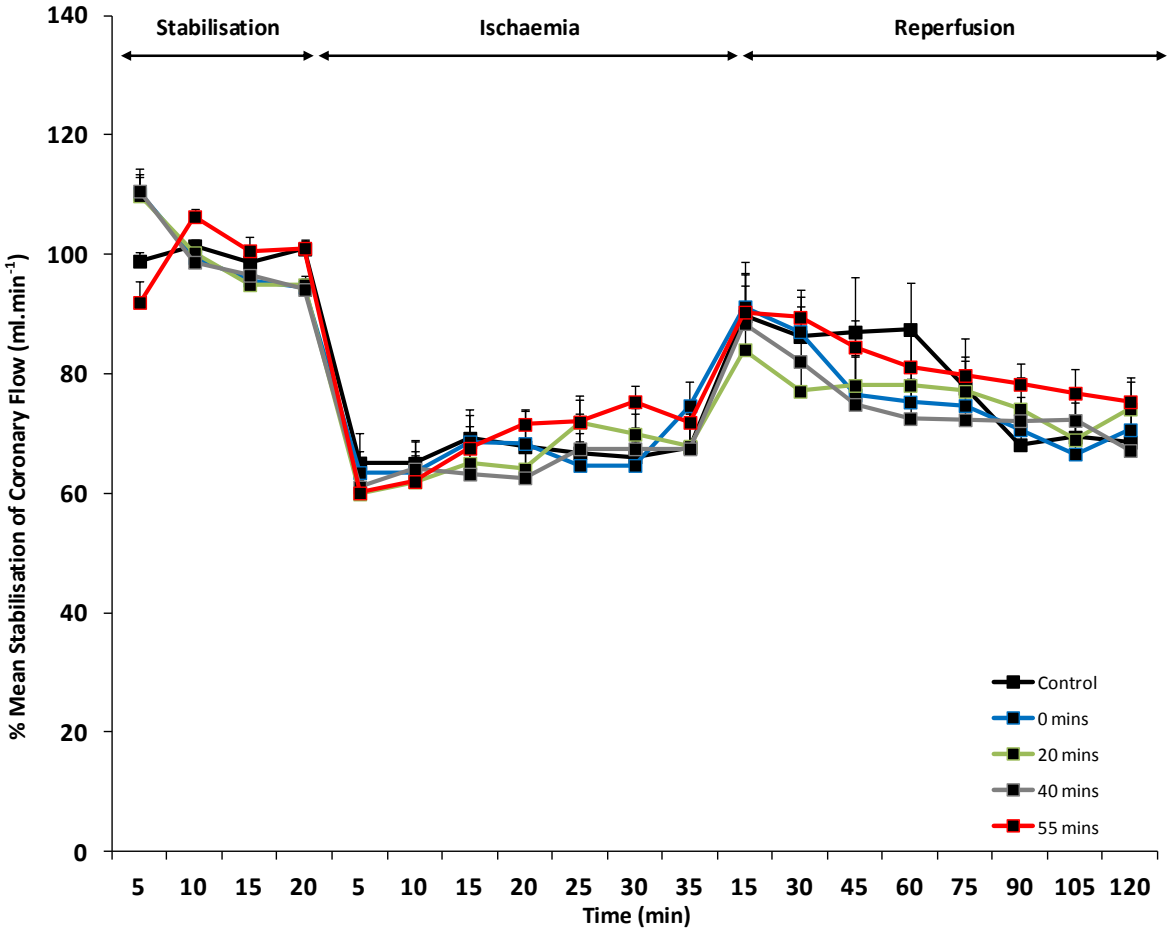


Figure 3.13: Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Administration of ipratropium (1 x 10^{-7} M) occurred at different time points, throughout stabilisation, ischaemia and reperfusion (0 mins), throughout ischaemia and reperfusion (20 mins), administered 15 minutes prior to the end of ischaemia and throughout reperfusion (40 mins) and throughout reperfusion only (55 mins).Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.5 Ipratropium administration at the onset of reperfusion

Having ascertained that ipratropium administration under conditions of ischaemia and reperfusion significantly increased myocardial injury, in subsequent experiments, it was decided that ipratropium would be administered at the onset of, and throughout, reperfusion. This was in order to identify the signalling mechanisms within reperfusion which were affected by ipratropium administration.

3.3.5.1 Infarct size to risk ratio (%) analysis

Figure 3.14 shows the observations from isolated perfused heart experiments where ipratropium (1×10^{-9} – 1×10^{-6} M) was administered at reperfusion following 35 minutes regional ischaemia. This data showed that ipratropium increases the development of infarct in a dose responsive manner. Infarct/Risk (%) (I/R (%)) was statistically different from control hearts (51.8 ± 3.0 %) at concentrations of 1×10^{-8} M and above (Table 3.2). A sub-maximal concentration of ipratropium (1×10^{-7} M) was used in all subsequent experiments.

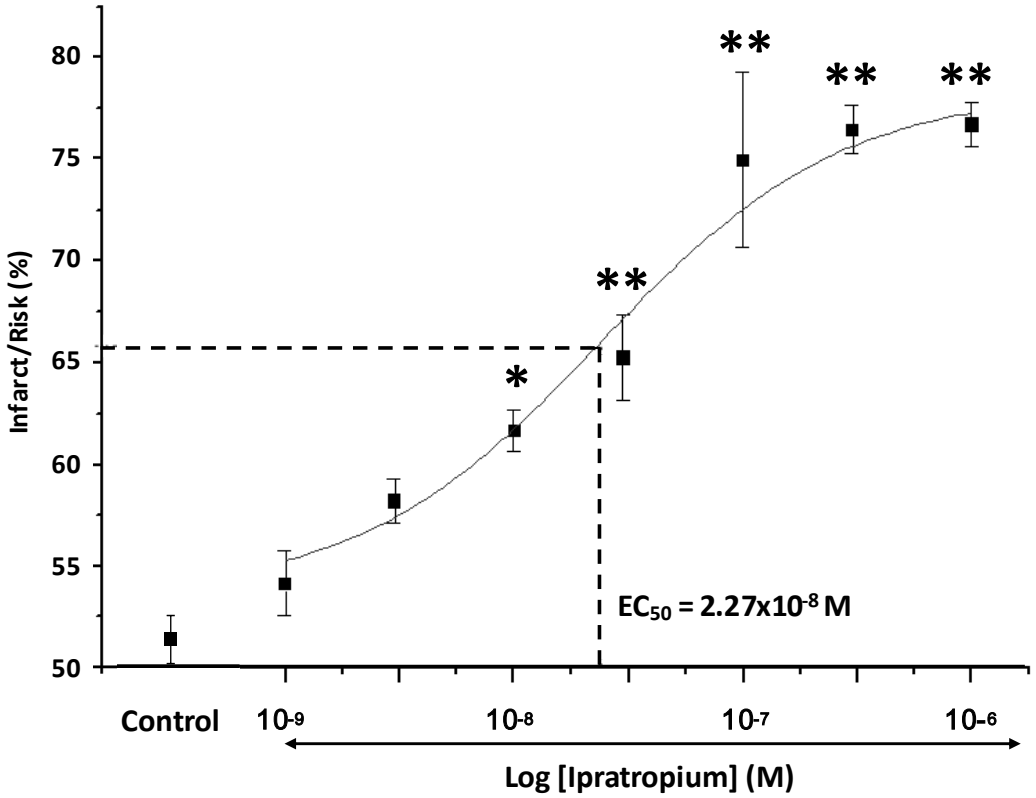


Figure 3.14: Infarct development in the risk zone after administration of different concentrations of Ipratropium (1 x 10^{-9} M – 1 x 10^{-6} M) when administered at the onset of, and throughout, reperfusion. Results are presented as infarct/risk ratio (Infarct/Risk %). Expressed as arithmetic mean + SEM. *p<0.05 and **p<0.01 vs. control, n=6.

		Concentration of ipratropium administered at the onset of reperfusion						
Group	Control	1x10^{-9} M	3x10^{-9} M	1x10^{-8} M	3x10^{-8} M	1x10^{-7} M	3x10^{-7} M	1x10^{-6} M
I/R (%)	51.8 ± 3.0%	54.2 ± 1.6%	58.3 ± 3.1%	61.7 ± 2.3% *	65.3 ± 4.6% **	74.3 ± 4.5% **	76.6 ± 2.6% **	76.8 ± 2.5% **

Table 3.2: Values for infarct development in the risk zone after administration of different concentrations of Ipratropium (1 x 10^{-9} M – 1 x 10^{-6} M) when administered at the onset of, and throughout, reperfusion. Results are presented as infarct/risk ratio (Infarct/Risk %). Expressed as arithmetic mean ± SEM. *p<0.05 and **p<0.01 vs. control, n=6.

3.3.5.2 Haemodynamic data analysis

All haemodynamic parameters were measured and expressed as previously described.

3.3.5.3 Left ventricular developed pressure (LVDP)

There was no significant difference in LVDP in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups ($p>0.05$) (Figure 3.15).

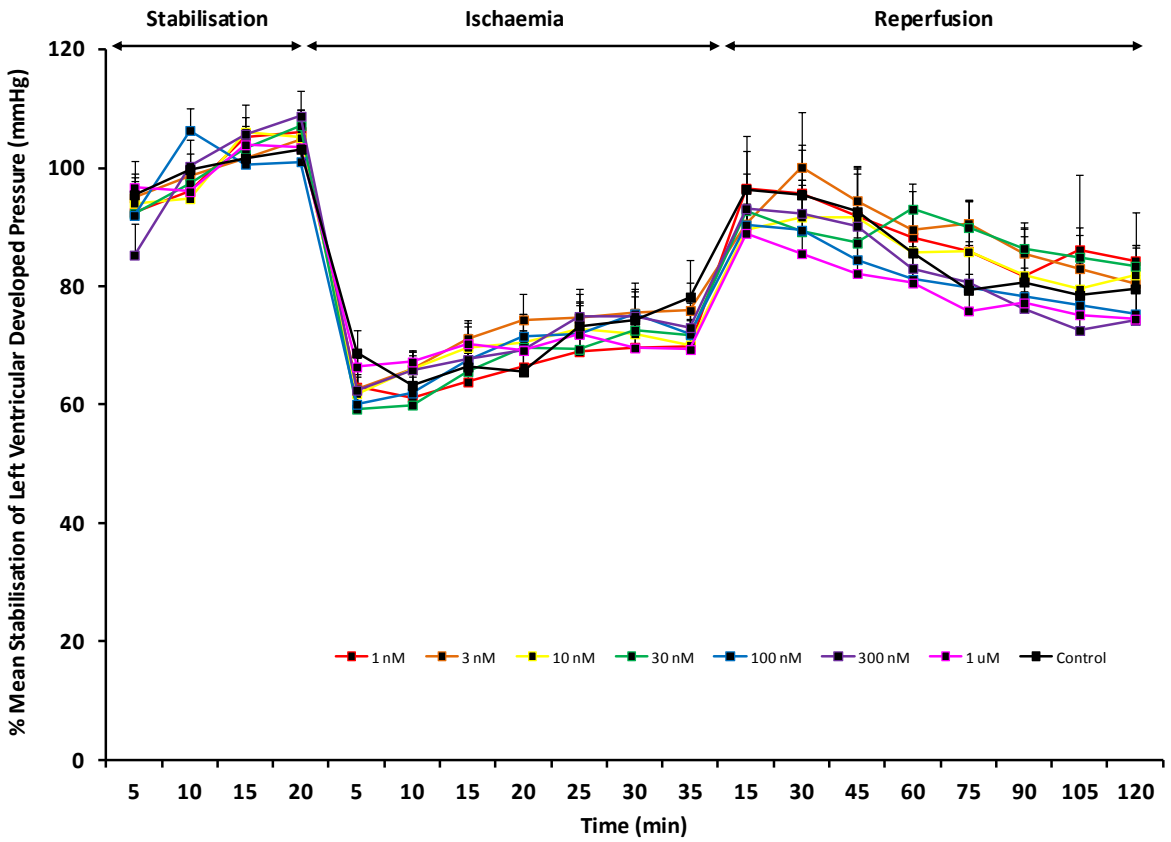


Figure 3.15: Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1 x 10^{-9} M – 1 x 10^{-6} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.5.4 Heart rate (HR)

There was no significant difference in HR in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups ($p>0.05$) (Figure 3.16).

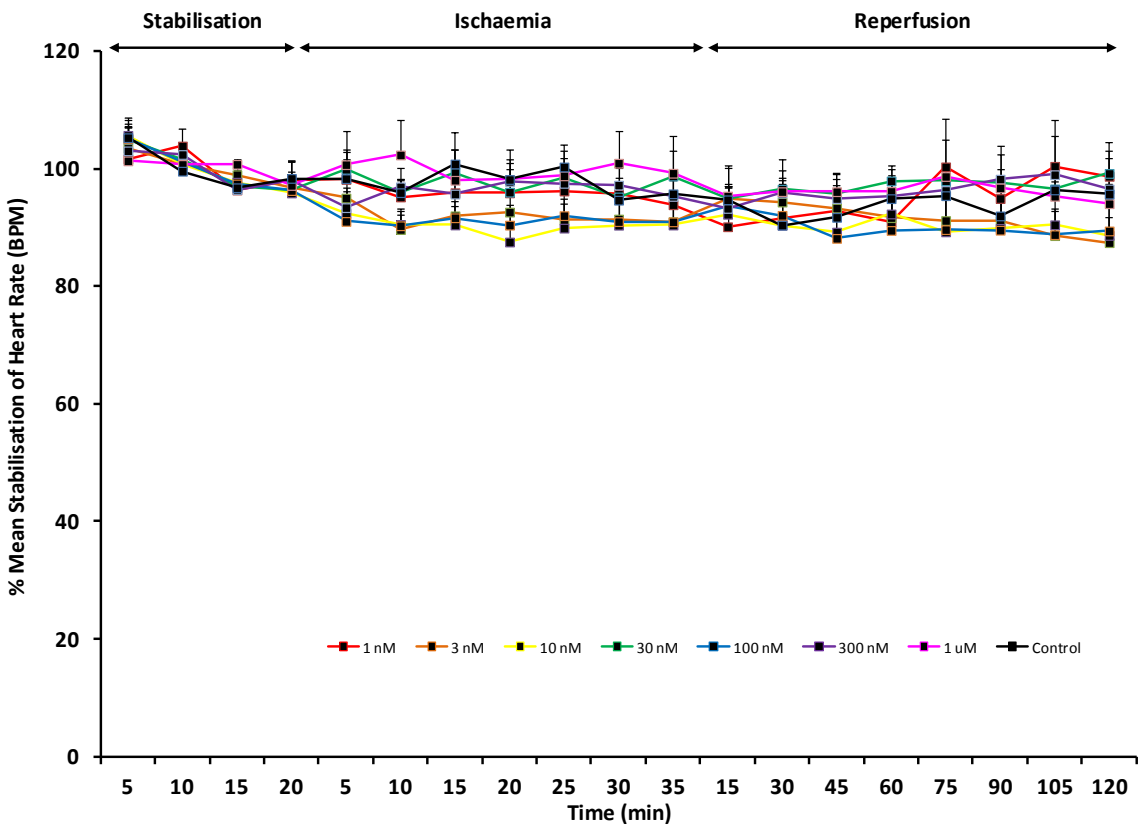


Figure 3.16: Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1 x 10^{-9} M – 1 x 10^{-6} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.5.5 Coronary flow (CF)

There was no significant difference in LVDP in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups ($p>0.05$) (Figure 3.17).

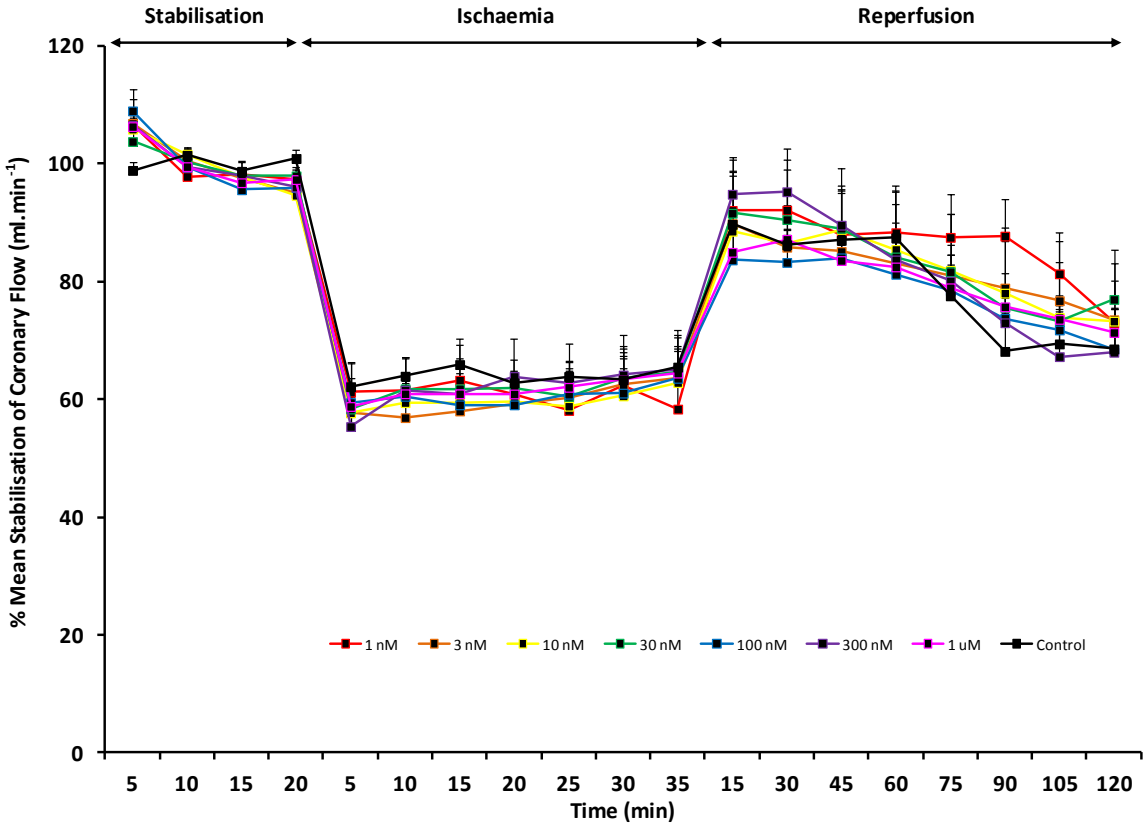


Figure 3.17: Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1 x 10^{-9} M – 1 x 10^{-6} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.6 Effect of ipratropium administration on cardiac myocyte viability with use of the MTT assay following hypoxia/reoxygenation

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) is reduced to purple formazan by mitochondrial dehydrogenase, and therefore provides a colorimetric assay for cellular viability. During these experiments, all groups were subjected to the H/R protocol (outlined in Section 2.4.1), with ipratropium or atropine administered at the onset of reoxygenation. Figure 3.18 shows percentage change in MTT reductase activity, indicative of myocyte viability, of the control in comparison with myocytes administered ipratropium or atropine (1×10^{-11} M – 1×10^{-6} M). Administration of ipratropium or atropine at the onset of re-oxygenation produced a significant reduction in cell viability in a concentration responsive manner, values shown in Table 3.3.

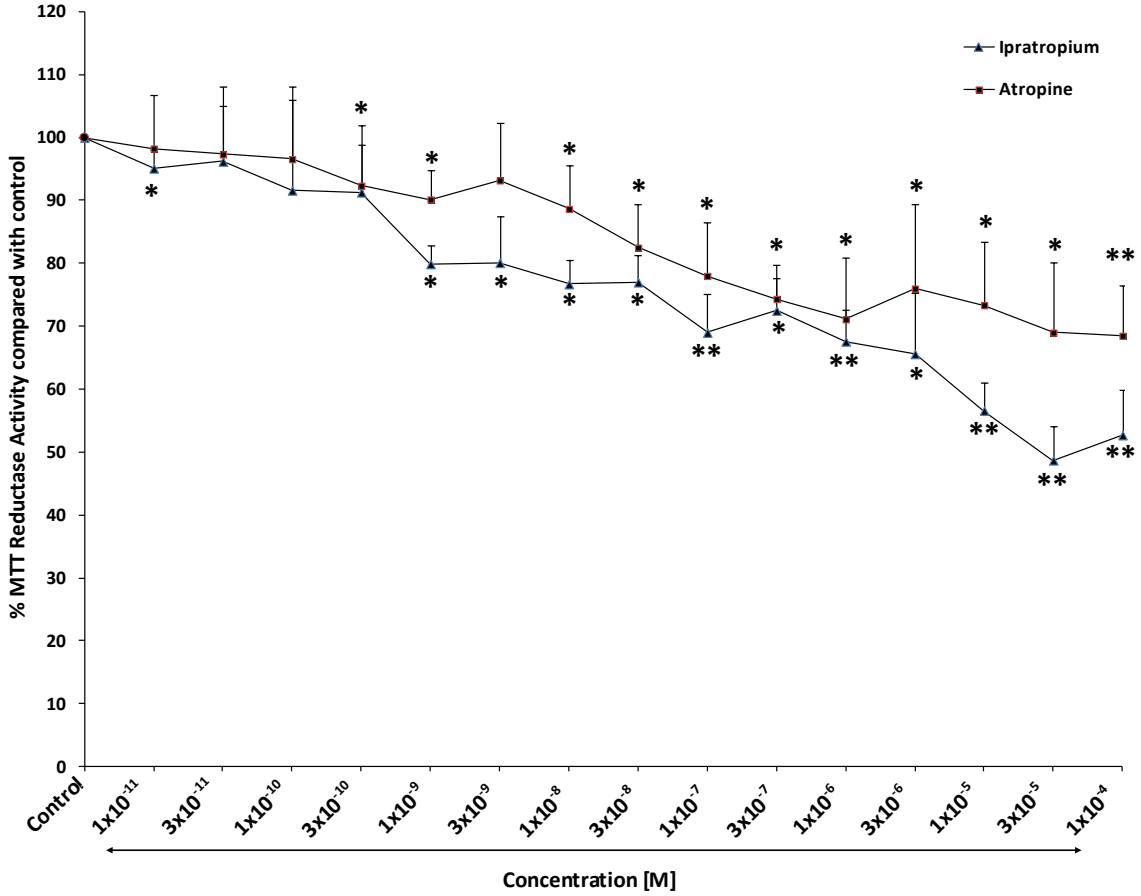


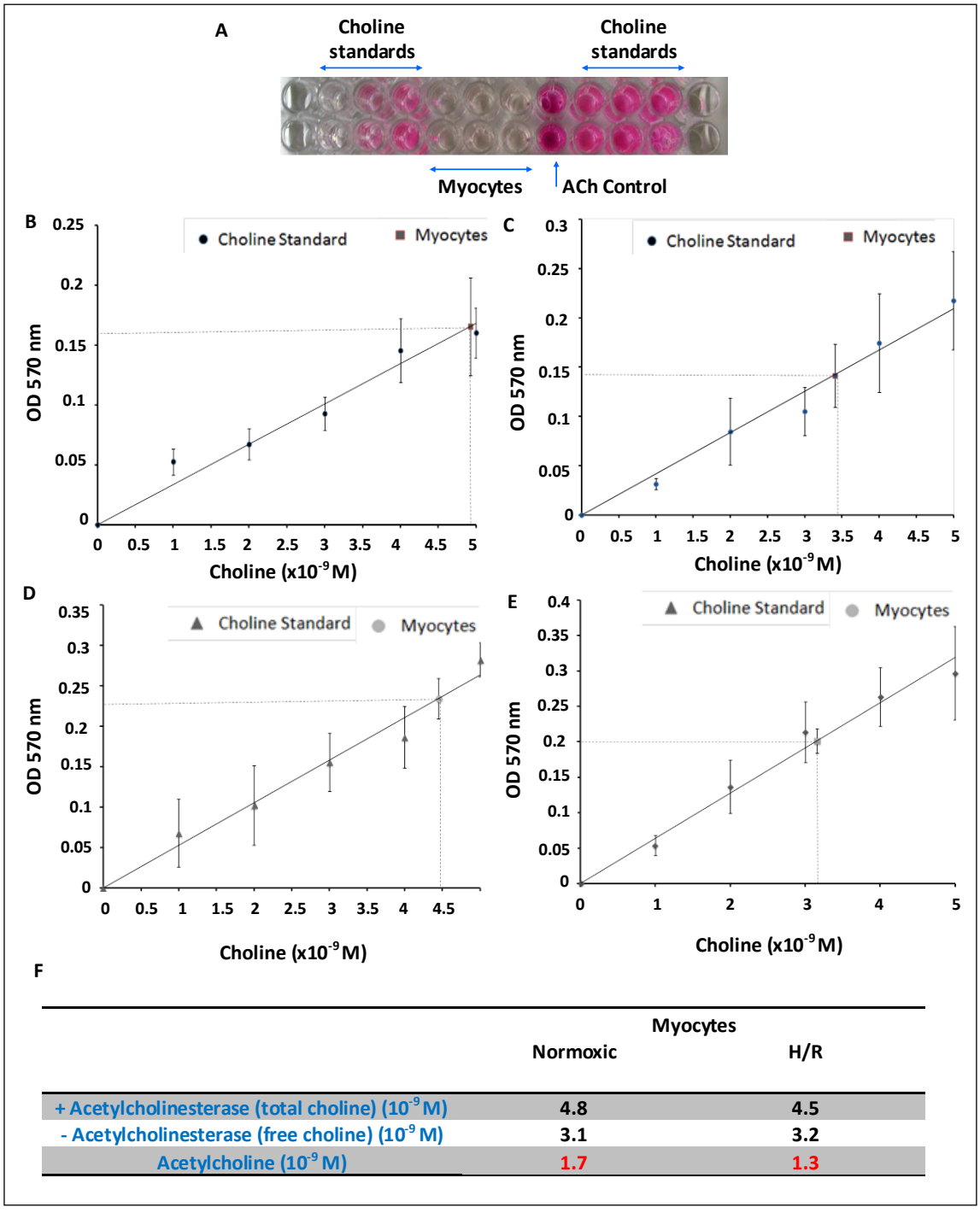
Figure 3.18: Assessment of myocyte viability via changes in MTT reductase activity in isolated rat ventricular myocytes following hypoxia/re-oxygenation. Administration of ipratropium or atropine (1×10^{-11} M – 1×10^{-4} M) occurred at the onset of, and throughout, re-oxygenation. All groups were subjected to hypoxia/re-oxygenation protocol. Values are mean + SEM, n=6. *p<0.05, **p<0.01 vs. control.

Group	MTT Reductase activity c/w Control (%) (Mean ± SEM)	
Control	100 ± 0.0	
	Ipratropium bromide:	**Atropine:**
1 x 10^{-11} M	95.1 ± 2.9 *	98.3 ± 8.6
3 x 10^{-11} M	96.2 ± 12.0	97.3 ± 7.8
1 x 10^{-10} M	91.6 ± 14.4	96.6 ± 11.6
3 x 10^{-10} M	91.3 ± 10.7	92.3 ± 6.5 *
1 x 10^{-9} M	79.9 ± 3.0 *	90.2 ± 4.6 *
3 x 10^{-9} M	80.1 ± 7.3 *	93.2 ± 9.1
1 x 10^{-8} M	76.7 ± 3.8 *	88.7 ± 6.9 *
3 x 10^{-8} M	77.0 ± 4.4 *	82.5 ± 6.9 *
1 x 10^{-7} M	69.0 ± 6.2 **	78.0 ± 8.5 *
3 x 10^{-7} M	72.5 ± 5.1 *	74.4 ± 5.5 *
1 x 10^{-6} M	67.6 ± 5.0 **	71.1 ± 9.7 *
3 x 10^{-6} M	65.6 ± 9.8 *	76.0 ± 13.5 *
1 x 10^{-5} M	56.6 ± 4.4 **	73.4 ± 10.2 *
3 x 10^{-5} M	48.7 ± 5.4 **	69.0 ± 11.2 *
1 x 10^{-4} M	52.7 ± 7.3 **	68.5 ± 8.0 **

Table 3.3: Values for myocyte viability via changes in MTT reductase activity in isolated rat ventricular myocytes following hypoxia/re-oxygenation. Administration of ipratropium or atropine (1 x 10^{-11} M – 1 x 10^{-4} M) occurred at the onset of, and throughout, re-oxygenation. All groups were subjected to hypoxia/re-oxygenation protocol. Values are mean ± SEM, n=6. *p<0.05, **p<0.01 vs. control.

3.3.7 Acetylcholine assay

In order to elucidate that the observed ipratropium induced injury involved disruption of muscarinic receptor signalling, it was necessary to ascertain whether levels of endogenous acetylcholine were present in the experimental model being utilised. With use of the Choline/Acetylcholine Assay kit, cell lysate acetylcholine levels in myocytes under normoxic conditions and those subjected to the H/R protocol were determined. The assay was conducted in the absence and presence of acetylcholinesterase in order to identify values of total and free choline to establish the concentration of acetylcholine in myocyte lysates (Figure 3.19). Choline standards were prepared to produce standard curves (**A**). Following colorimetric analysis at 450 nm, these allowed quantification of acetylcholine levels. Under normoxic conditions (**B** & **C**), levels of acetylcholine were found to be 1.7×10^{-9} M. In cell lysates from myocytes subjected to the H/R protocol (**D** & **E**), the concentration of acetylcholine was 1.3×10^{-9} M. Calculations are provided in the table (**F**).



F

	Myocytes	
	Normoxic	H/R
+ Acetylcholinesterase (total choline) (10^{-9} M)	4.8	4.5
- Acetylcholinesterase (free choline) (10^{-9} M)	3.1	3.2
Acetylcholine (10^{-9} M)	1.7	1.3

Figure 3.19: Endogenous levels of acetylcholine as determined by choline/acetylcholine assay, myocytes plated out at 1 x 10^6 cells.ml^{-1}. **A**, representative 96 well plate in which the acetylcholine assay has been carried out. Panels **B** and **C**: Levels of total choline (**B**, 4.92 x 10^{-9} M ± 0.04) and free choline (**C**, 3.41 x 10^{-9} M ± 0.03) in normoxic, untreated adult rat ventricular myocytes. Panels **D** and **E**: Levels of total choline (**D**, 4.49 x 10^{-9} M ± 0.03) and free choline (**E**, 3.16 x 10^{-9} M ± 0.02) in untreated adult rat ventricular myocytes subjected to hypoxia/re-oxygenation protocol. Expressed as arithmetic mean ± SEM, n=5.

3.3.8 Effect of acetylcholine ± ipratropium treatment on isolated perfused rat hearts

Having identified endogenous levels of acetylcholine, it was postulated that administration of exogenous acetylcholine may have an effect on ipratropium induced myocardial injury. Further isolated perfused rat heart experiments were conducted with the use of ipratropium ± acetylcholine (both 1 x 10^{-7} M) and atropine ± acetylcholine (both 1 x 10^{-7} M) to further identify the involvement of muscarinic signalling.

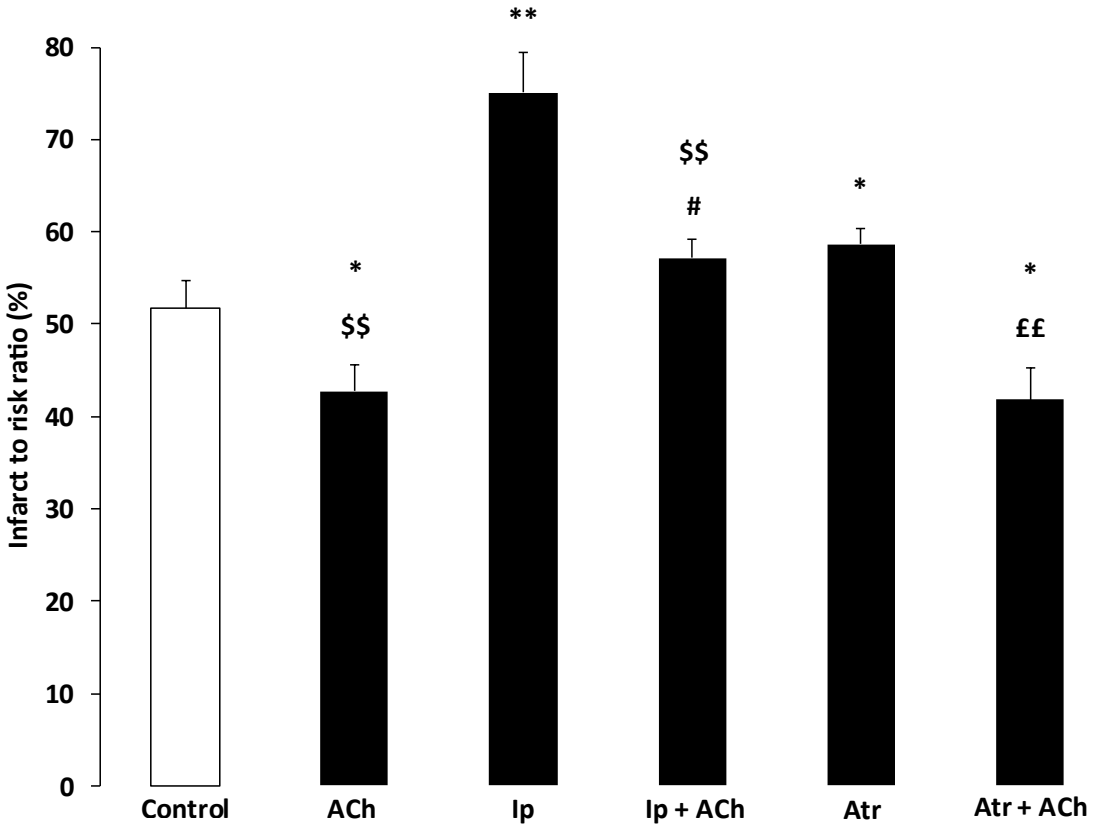


Figure 3.20: The effect of 1 x 10^{-7} M acetylcholine (ACh) administration at reperfusion on ipratropium (1 x 10^{-7} M) and atropine (1 x 10^{-7} M) induced myocardial injury in the isolated perfused rat heart. Results are presented as infarct/risk ratio (I/R%). Mean + SEM, n=6, *p<0.05, **p<0.01 vs. control, $$p<0.01 vs. ipratropium, #p<0.05 vs. Ach, ££p<0.01 vs. atropine.

3.3.8.1 Ipratropium induced exacerbation of myocardial I/R injury is significantly abrogated by acetylcholine in the isolated perfused rat heart

The results presented in Figure 3.20 show the effect of acetylcholine (1×10^{-7} M) administration at reperfusion. Acetylcholine significantly reduced infarct size in comparison with the control (42.7 ± 2.9 % (ACh, 1×10^{-7} M) vs. 51.8 ± 3.0 % (control), $p<0.05$). The observed increase in infarct size due to ipratropium treatment was significantly attenuated when administered in conjunction with acetylcholine. Atropine (1×10^{-7} M) administration at reperfusion was also shown to significantly increase infarct development in comparison with the control (58.64 ± 1.7 % (Atr, 1×10^{-7} M) vs. 51.8 ± 3.0 % (control), $p<0.05$), which was also significantly attenuated upon acetylcholine administration.

3.3.8.2 Haemodynamic parameters

All haemodynamic parameters were measured and expressed as previously described.

3.3.8.3 Left ventricular developed pressure (LVDP)

There was no significant difference in LVDP in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M), atropine (1 x 10^{-7} M) or the groups where ipratropium and atropine were co-administered with acetylcholine (1 x 10^{-7} M) for any of the experimental groups in comparison with the untreated control. However, at 60, 75, 90, 105 and 120 mins time points, acetylcholine significantly increased LVDP in comparison with the control (*p<0.05, **p<0.01, data presented in table 3.4) (Figure 3.21).

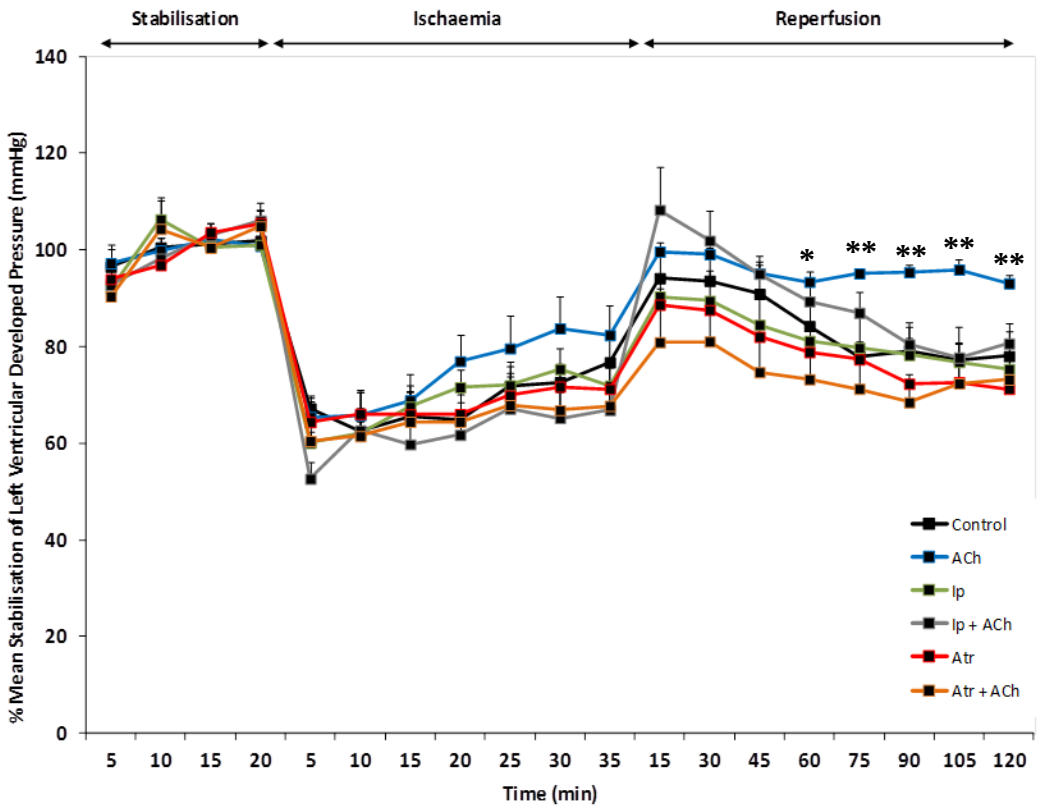


Figure 3.21: Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium (1 x 10^{-7} M), atropine (1 x 10^{-7} M) and acetylcholine (1 x 10^{-7} M) ± ipratropium or atropine (both 1 x 10^{-7} M) were administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups.*p<0.05 and ** p<0.01 vs. Control.

3.3.8.4 Heart Rate (HR)

There was no significant difference in HR in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M), atropine (1 x 10^{-7} M), acetylcholine (1 x 10^{-7} M) or the groups where ipratropium and atropine were co-administered with acetylcholine (all drugs 1 x 10^{-7} M) for any of the experimental groups in comparison with the untreated control at any time point (Figure 3.22).

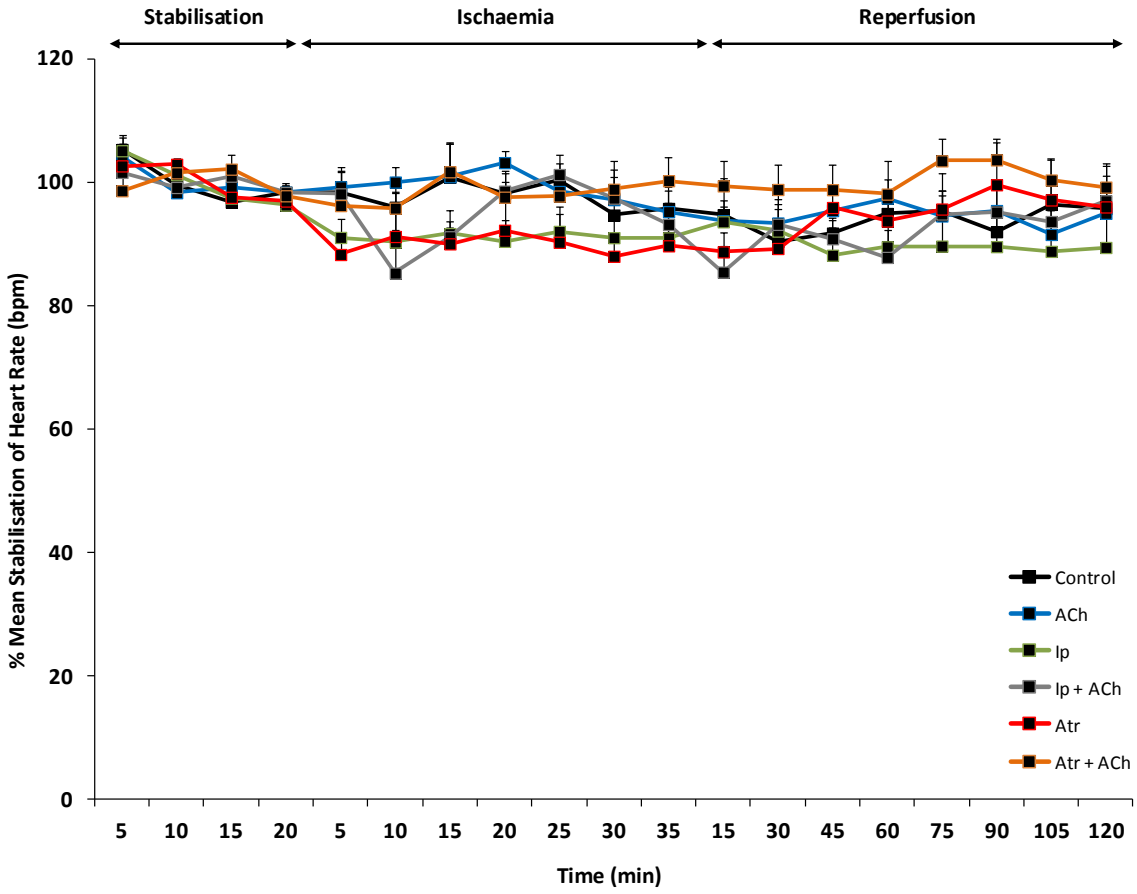


Figure 3.22: Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium (1 x 10^{-7} M), atropine (1 x 10^{-7} M) and acetylcholine (1 x 10^{-7} M) ± ipratropium or atropine (both 1 x 10^{-7} M) were administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.8.5 Coronary flow (CF)

There was no significant difference in CF in comparison with the control with hearts treated with ipratropium (1 x 10^{-7} M), atropine (1 x 10^{-7} M), acetylcholine (1 x 10^{-7} M) or the groups where ipratropium and atropine were co-administered with acetylcholine (all drugs 1 x 10^{-7} M) for any of the experimental groups in comparison with the untreated control at any time point (Figure 3.23).

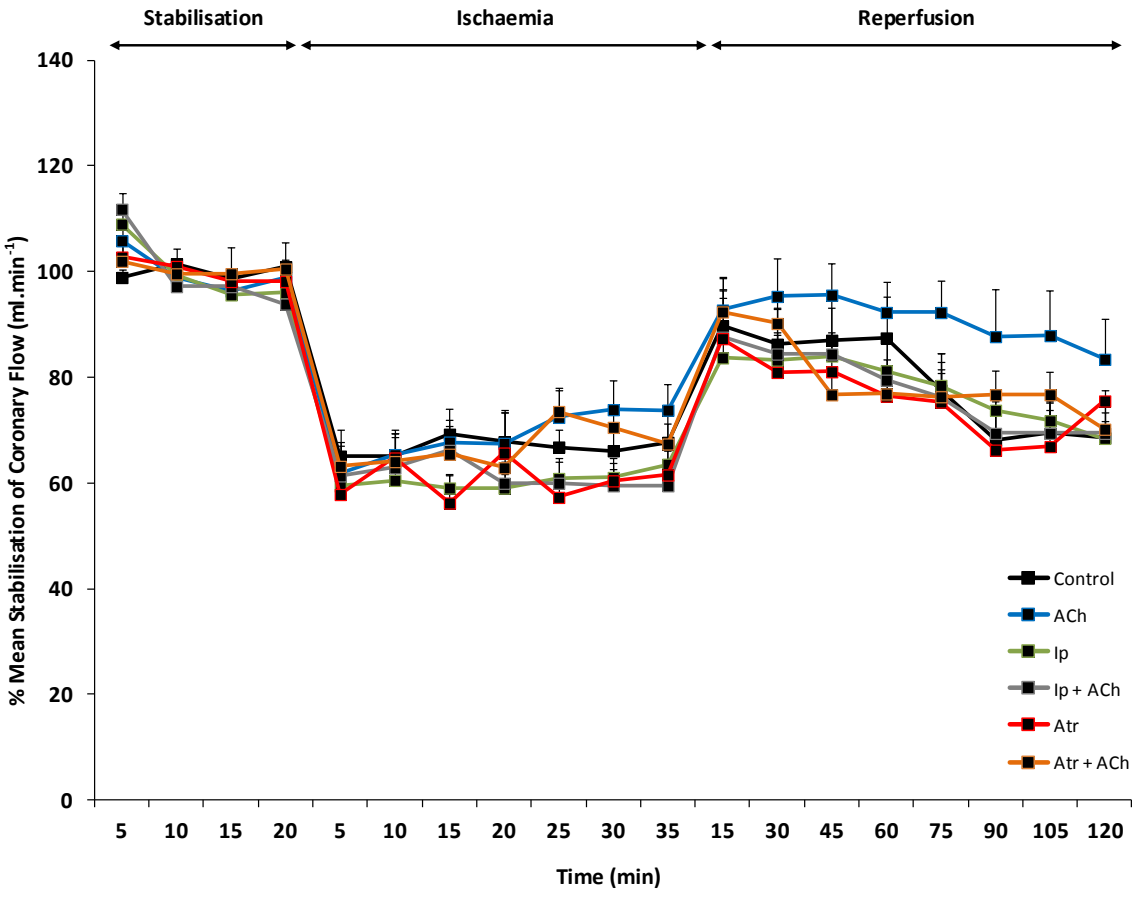


Figure 3.23: Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium (1 x 10^{-7} M), atropine (1 x 10^{-7} M) and acetylcholine (1 x 10^{-7} M) ± ipratropium or atropine (both 1 x 10^{-7} M) were administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.9 Assessment of MTT reductase activity following acetylcholine ± ipratropium treatment

The cell viability assay was conducted in the presence and absence of acetylcholine (1 x 10^{-7} M) ± ipratropium (1 x 10^{-7} M) or atropine (1 x 10^{-7} M) (Figure 3.24). Treatment with acetylcholine significantly increased cell viability (108.0 ± 4.6 %, (ACh, 1 x 10^{-7} M) vs. 100 ± 0.0 % (control), $p<0.05$) in comparison with the control. Concomitant treatment with ipratropium and acetylcholine significantly increased viability in comparison with myocytes treated with ipratropium alone (88.7 ± 4.5 % (Ip + Ach) vs. 73.0 ± 2.1 % (Ip, 1 x 10^{-7} M), $p<0.05$). A significant decrease in myocyte viability was observed in the group treated with atropine (81.3 ± 8.3 % (Atr, 1 x 10^{-7} M) vs. 100 ± 0.0 % (control), $p<0.05$). In comparison with the group treated with both atropine and acetylcholine, there was a small increase in viability in comparison with the atropine group, however this was statistically insignificant ($p>0.05$).

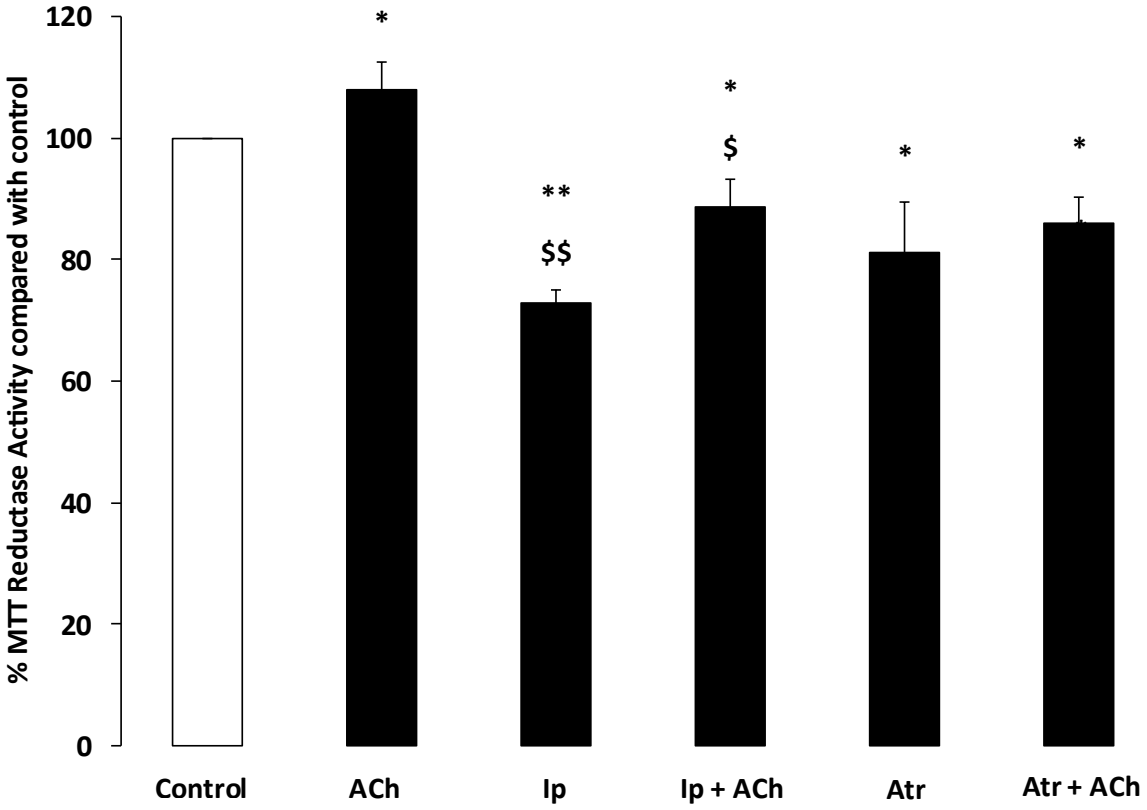


Figure 3.24: Effect of ipratropium (1 x 10^{-7} M), atropine (1 x 10^{-7} M) and acetylcholine (1 x 10^{-7} M) ± ipratropium or atropine on MTT reductase activity in isolated rat ventricular myocytes following hypoxia/re-oxygenation, normalised as a percentage of the untreated control. Administration of ipratropium (Ip 1 x 10^{-7} M), atropine (Atr, 1 x 10^{-7} M) and acetylcholine (ACh, 1 x 10^{-7} M) occurred at the onset of, and throughout, re-oxygenation. Values are mean + SEM, n=6. *p<0.05, **p<0.01 vs. Control, $p<0.05, $$p<0.01 vs. ACh (1 x 10^{-7} M).

3.3.10 Assessment of apoptosis and necrosis in primary cardiac myocytes following hypoxia/re-oxygenation and ipratropium treatment

Additional experiments were conducted to identify whether the observed ipratropium induced increase in I/R (%) and reduction in cell viability was due to apoptosis or necrosis. For all experiments, normoxic controls were carried out in order to verify the H/R protocol (as described in section 2.4.1). A 40% decrease in viable myocytes following H/R in comparison with the normoxic control was required to ascertain that the H/R protocol had been successful.

Viable, necrotic and apoptotic myocytye populations were recorded via flow cytometry from cardiac myocytes exposed to ipratropium treatment (1×10^{-9} M – 1×10^{-6} M) throughout re-oxygenation (Figures 3.25 and 3.26). Viable cells were calculated by the percentage of cells able to metabolise C_{-12} resazurin and, within metabolically active, viable cells, resazurin is reduced to C_{-12}-resorufin which is fluorescent on the red spectrum, thereby enabling identification on the flow cytometer. At all concentrations of ipratropium tested (1×10^{-9} – 1×10^{-6} M), there was a significant decrease in myocyte viability in comparison with the untreated, H/R control (Figure 3.25, values in Table 3.4, $p<0.01$ for all concentrations of ipratropium in comparison with control).

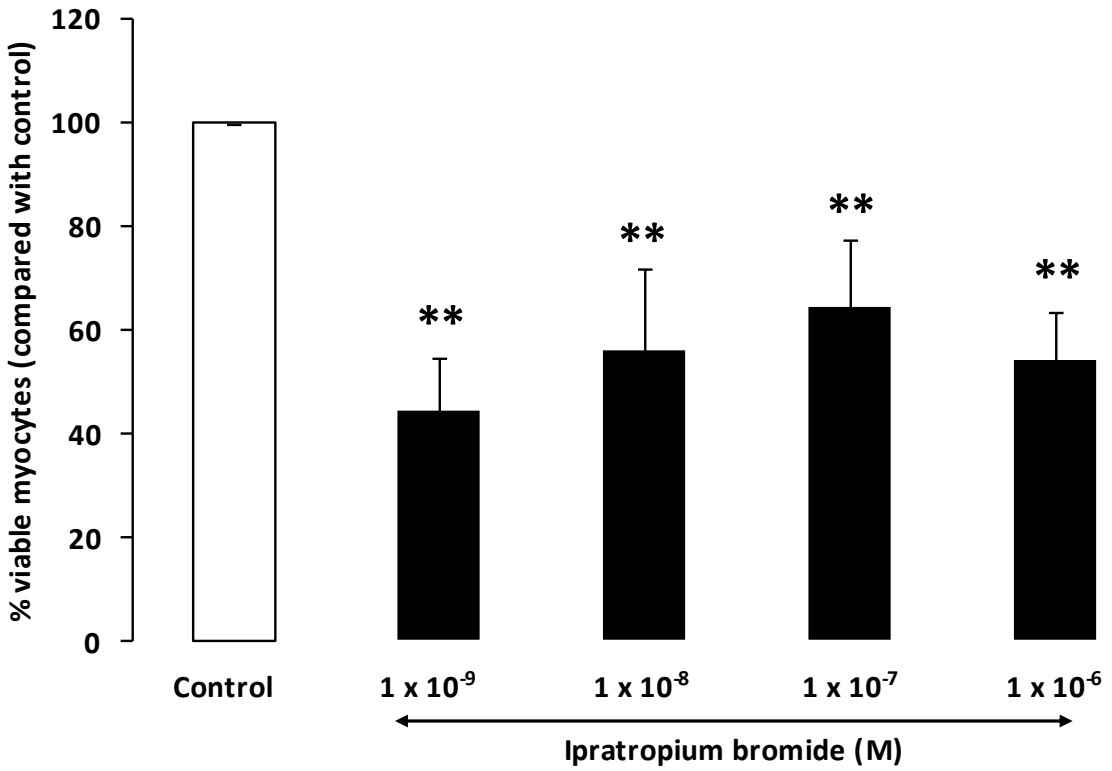


Figure 3.25: Assessment of viable adult rat ventricular myocytes following H/R protocol and ipratropium administration.**p<0.01 in comparison with untreated control, values are mean + SEM (n=10).

Group	Control	Ipratropium bromide (M)			
		1 x 10^{-9}	1 x 10^{-8}	1 x 10^{-7}	1 x 10^{-6}
Viable myocytes (%)	**100 ± 0.0**	**44.5±10.2****	**56.1±15.9****	**64.4±13.2****	**54.0±9.3****

Table 3.4: Values for flow cytometric assessment of viable adult rat ventricular myocytes following H/R protocol and ipratropium administration. **p<0.01 in comparison with untreated control, values are mean ± SEM (n=10).

Apoptosis and necrosis were estimated by Annexin V and SYTOX® Green positive myocytes, respectively. All concentrations of ipratropium tested, caused a significant increase in apoptosis (Annexin V positive) in comparison with the untreated control (100 ± 0.0 %, control vs. 169.8 ± 19.7 % (1 x 10^{-9} M), 160.9 ± 23.3 % (1 x 10^{-8} M), 139.9 ± 18.1 % (1 x 10^{-7} M) and 153.1 ± 17.9 % (1 x 10^{-6} M), all $p<0.05$. In addition ipratropium administration caused a significant increase in necrosis (SYTOX® Green positive) above the control group at 1 x 10^{-8} M, 1 x 10^{-7} M and 1 x 10^{-6} M ipratropium (100 ± 0.0 %, control vs. 142.6 ± 15.5 % (1 x 10^{-8} M), 141.6 ± 23.9 % (1 x 10^{-7} M) and 140.9 ± 17.7 % (1 x 10^{-6} M), all $p<0.05$).

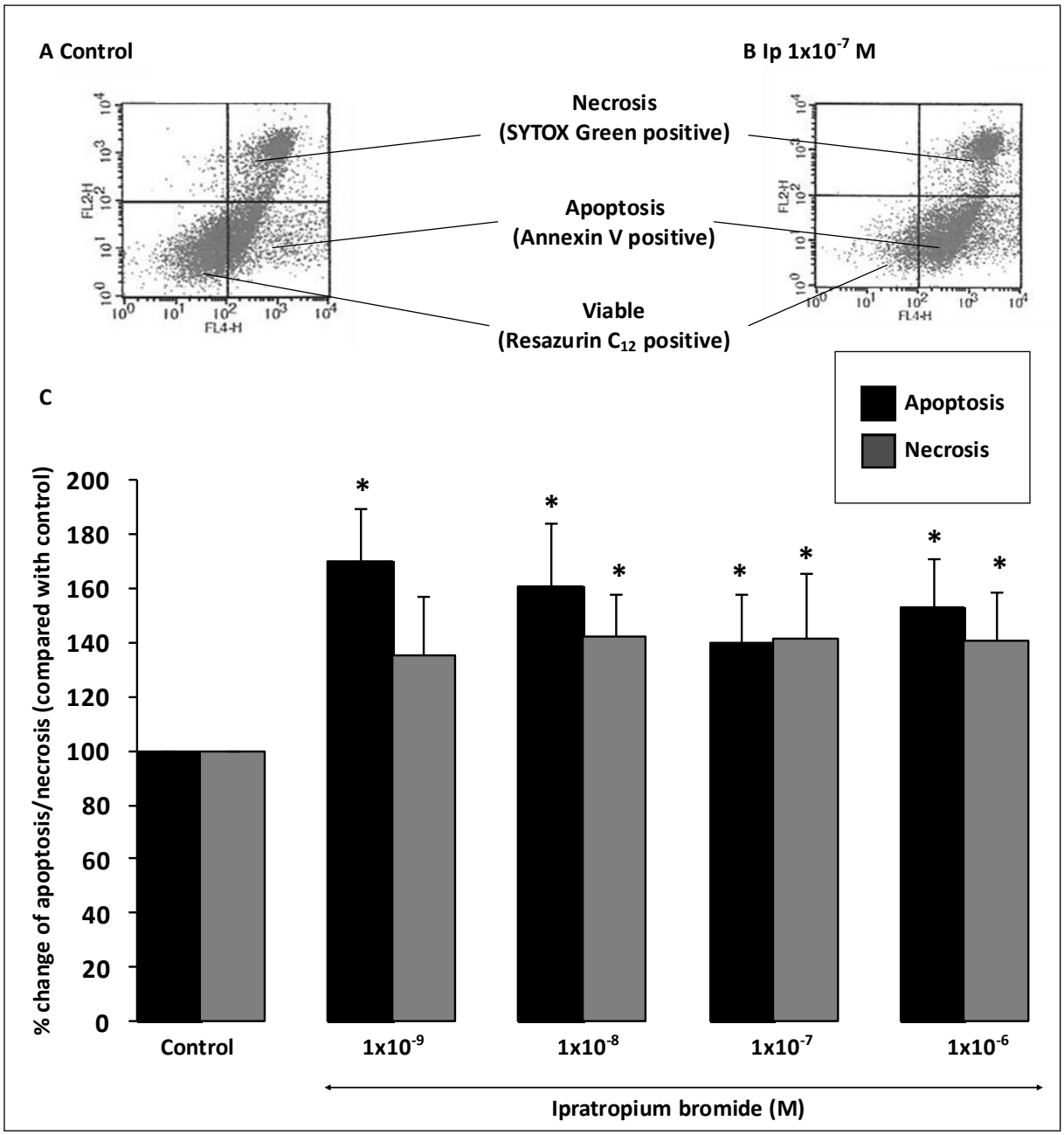


Figure 3.26: Assessment of apoptosis and necrosis in adult rat ventricular cardiac myocytes following H/R protocol and ipratropium treatment. **A** and **B**, original representative flow cytometric scatter graphs presenting myocytes subjected to hypoxia and re-oxygenation in the absence (**A**) and presence (**B**) of ipratropium to indicate the differentiation between necrotic, apoptotic and viable myocytes. **C**. Depicts normalised data for apoptotic and necrotic myocyte populations expressed as percentage of untreated control. *p<0.05 in comparison with respective untreated control, values are mean + SEM (n=10).

3.3.11 Assessment of cleaved caspase-3 levels following hypoxia/ re-oxygenation in primary cardiac myocytes

Having established an increase in apoptotic death, following ipratropium administration at the onset of re-oxygenation, the involvement of caspase-3, a pivitol caspase in the apoptotic machinery was investigated. In order to ascertain that the H/R protocol had been successful, a significant increase in caspase-3 in the H/R control group in comparison with the normoxic group was required (as shown in Figure 3.27). In order to display the data as a single channel histogram, the FL1-H channel was used. The FL1-H flow channel was used as it was appropriate for the AlexaFluor488® conguated antibody for cleaved caspase-3 (with excitation laser lines of 488 nm) and this channel allowed for maximum excitation of 495 nm and maximum emission of 519 nm.

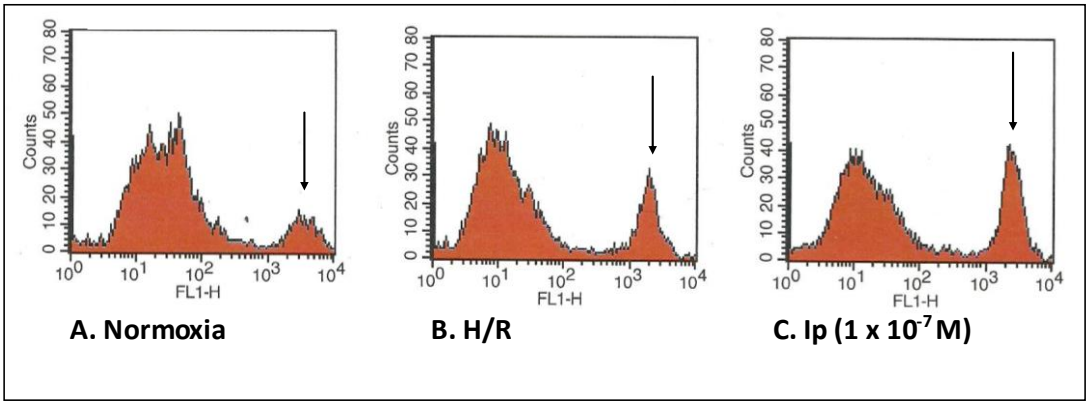


Figure 3.27: Representative flow cytometric scatter plots to show levels of activated cleaved caspase-3 in normoxic cardiac myocytes (**A**), following hypoxia/re-oxygenation protocol (**B**) and with ipratropium administration at the onset of re-oxygenation (**C**). Arrows indicate the peaks which represent cleaved caspase-3 levels.

3.3.11.1 Assessment of caspase-3 levels in primary cardiac myocytes following hypoxia/re-oxygenation and acetylcholine ± ipratropium treatment

Levels of cleaved caspase-3 were evaluated by flow cytometry. The data are presented in Figure 3.28. Panel **A** shows a representative histogram from the flow cytometer. **B** shows that exposure of ventricular cardiac myocytes to ipratropium throughout re-oxygenation is associated with an increase in levels of cleaved caspase-3 (Asp 175), an effect which was significant when ipratropium was administered at 1×10^{-7} M and 1×10^{-6} M (100 ± 0.0%, control vs. 128.7 ± 5.82% (1×10^{-7} M), $p<0.05$, 147.5 ± 12.8% (1×10^{-6} M), $p<0.01$. Addition of acetylcholine (1×10^{-7} M) (**C**) significantly decreased the level of cleaved caspase-3 compared with the control (70.2 ± 10.5% vs. 100 ± 0.0% (control), $p<0.05$). Administration of atropine also produced a significant increase in caspase-3 levels in comparison with the control (119.8 ± 6.73% vs. 100 ± 0.0% (control), $p<0.05$). However, the co-administration of ipratropium or atropine with acetylcholine produced effects which, although showed a decrease in cleaved caspase-3, were statistically insignificant in comparison with the respective groups treated with ipratropium or atropine alone.

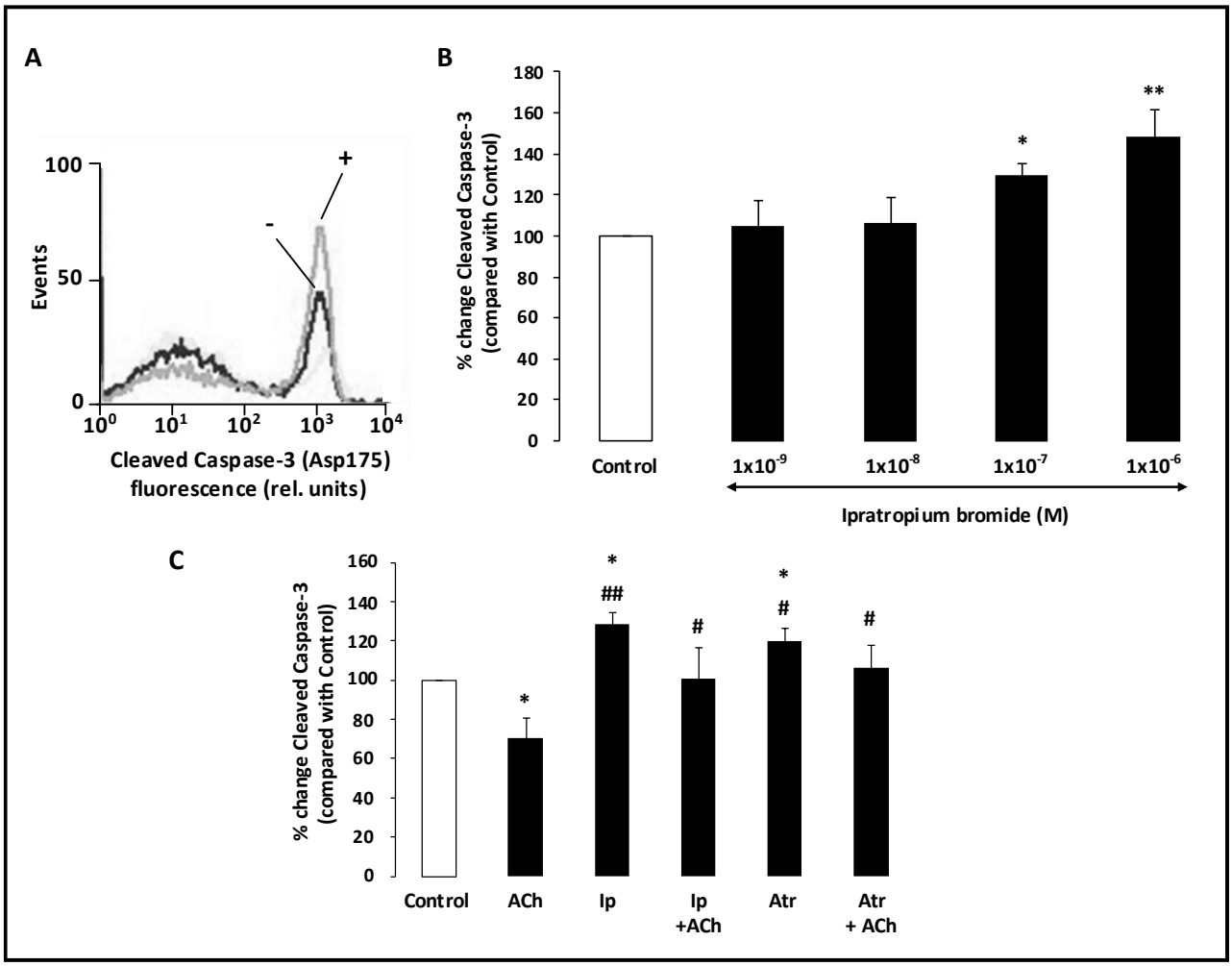


Figure 3.28: Effect of ipratropium (Ip), atropine (Atr) and acetylcholine (Ach) on cleaved caspase-3 levels in ventricular myocytes, following hypoxia and re-oxygenation. Ipratropium, atropine and acetylcholine administered throughout re-oxygenation. **A**. Original histogram depicting cleaved caspase-3 fluorescence (Alexa Fluor 488® conjugate) in myocytes following 2 hours hypoxia and exposure to re-oxygenation without (-) and with (+) 1 x 10^{-7} M ipratropium. **B**. Effect of ipratropium (1 x 10^{-9} M – 1 x 10^{-6} M) on caspase-3 levels of normalised histogram data expressed as percentage of untreated control (white bar). **C**. Effect of acetylcholine (ACh, 1 x 10^{-7} M) ± ipratropium (Ip, 1 x 10^{-7} M) and atropine (Atr, 1 x 10^{-7} M) on levels of cleaved caspase-3 in isolated rat ventricular myocytes following hypoxia/re-oxygenation, as determined by flow cytometric analysis. Expressed as arithmetic means, normalised to untreated control, + SEM, (n=6), *p<0.05 and **p<0.01 vs. respective control. For **C**, #p<0.05 and ##p<0.01 vs. ACh (1 x 10^{-7} M).

3.3.12 Isolated perfused rat heart experiments with DEVD ± ipratropium treatment

The identification of increases in both apoptosis and caspase-3, as established via flow cytometry, led to the conclusion that caspase-dependent apoptotic death was involved in ipratropium induced myocardial injury following simulated I/R. In order to more fully elucidate the involvement of caspases, a potent caspase-3 inhibitor (Z-DEVD-FMK), known to significantly attenuate injury following I/R (AL-Rajaibi et al. 2008), was co-administered with ipratropium.

3.3.12.1 DEVD significantly attenuates ipratropium induced myocardial injury following I/R in the perfused rat heart

The involvement of caspase-3 in myocardial cell death following ipratropium treatment was assessed in perfused hearts via the administration of Z-DEVD-FMK (DEVD, 7 x 10^{-9} M) in conjunction with ipratropium (1 x 10^{-7} M) at the onset of reperfusion. These data are presented in Figure 3.29. Administration of DEVD produced a significant reduction in I/R (%) in comparison with the untreated control (51.8 ± 3.0%, control vs. 29.7 ± 4.7%, DEVD, $p<0.01$). As previously demonstrated, the group treated with 1 x 10^{-7} M ipratropium induced a significant increase in infarct/risk (%) of 75.1 ± 4.3%. In the group treated with both DEVD and Ipratropium, infarct development was significantly inhibited in comparison with both the untreated control and ipratropium (1 x 10^{-7} M) treatment group (36.7 ± 3.7% (DEVD + Ip), vs. 51.8 ± 3.0% and 75.1 ± 4.3% respectively, both $p<0.01$).

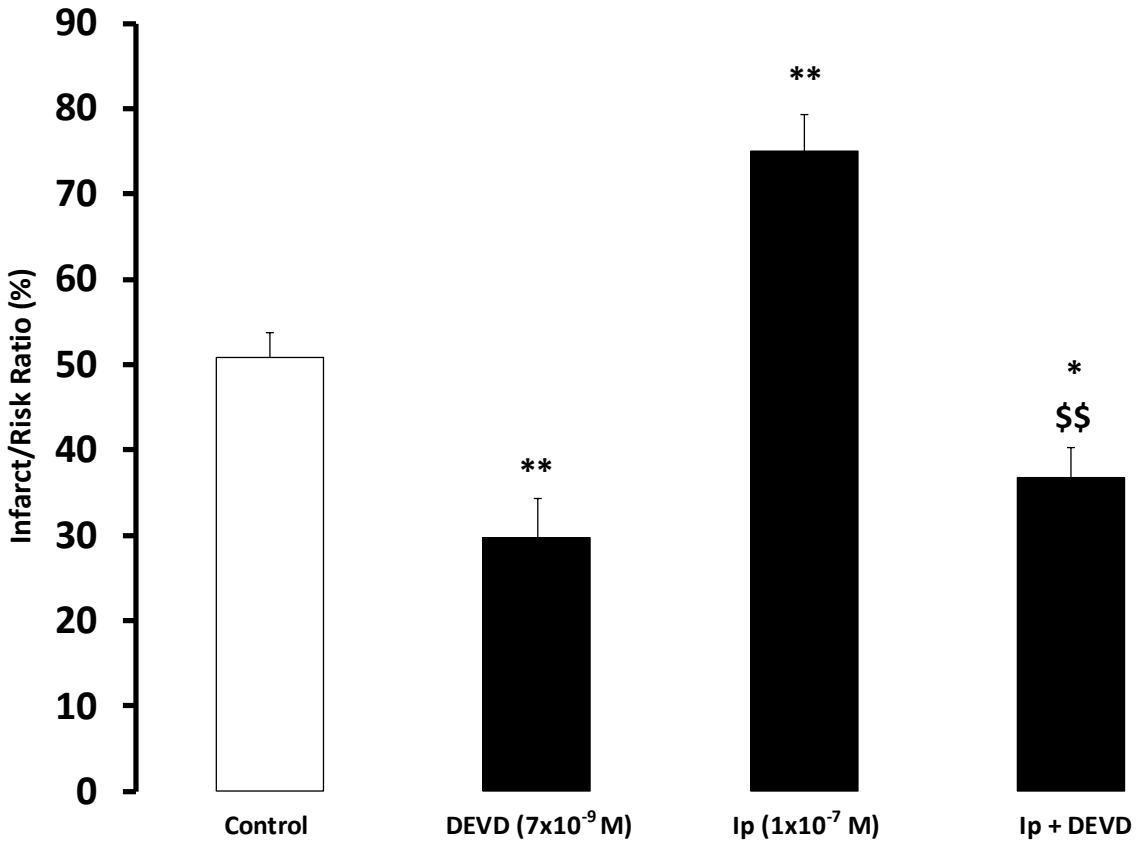


Figure 3.29: Infarct development in the risk zone following Z-DEVD-FMK (7 x 10^{-9} M) treatment ± ipratropium (1 x 10^{-7} M) in isolated perfused rat heart. DEVD and ipratropium both administered at the onset of reperfusion. Results are presented as infarct/risk (%).* $p<0.05$ (36.7 ± 3.7% (DEVD + ipratropium) and ** $p<0.01$ (29.7 ± 4.7% (DEVD) and 75.1 ± 4.3% (ipratropium) vs. control (51.8 ± 3.0%). $$p<0.01 (36.7 ± 3.7% (DEVD + ipratropium) vs. 75.1 ± 4.3% (ipratropium)). Mean + SEM, n=6.

3.3.12.2 Haemodynamic parameters

All haemodynamic parameters were measured and expressed as previously described (Section 2.3.3).

3.3.12.3 Left ventricular developed pressure (LVDP)

There was no significant difference in LVDP in comparison with the control with hearts subjected to ipratropium (1 x 10^{-7} M) or DEVD (7 x 10^{-8} M) ± ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups (Figure 3.30).

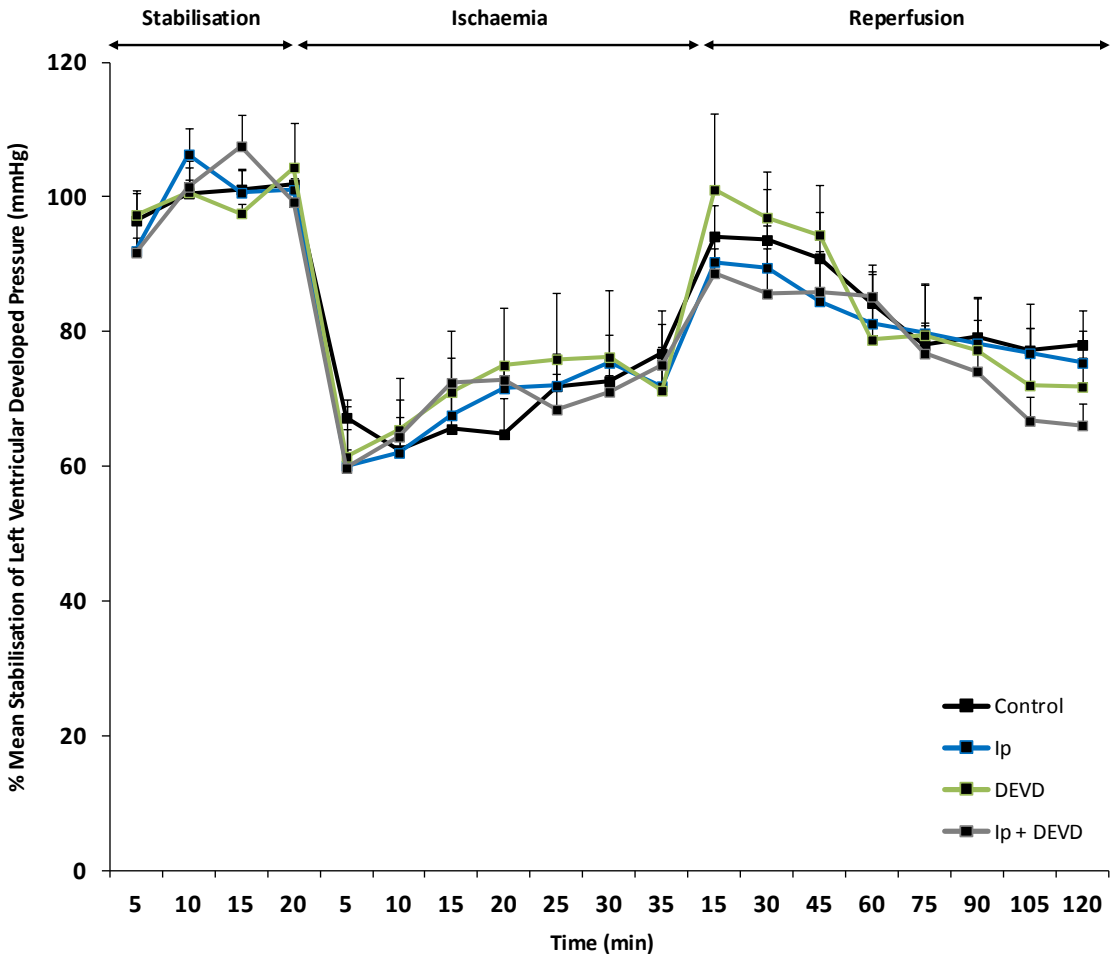


Figure 3.30: Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1 x 10^{-7} M) ± DEVD (7 x 10^{-8} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.12.4 Heart rate (HR)

There was no significant difference in HR in comparison with the control with hearts subjected to ipratropium (1 x 10^{-7} M) or DEVD (7 x 10^{-8} M) ± ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups (Figure 3.31).

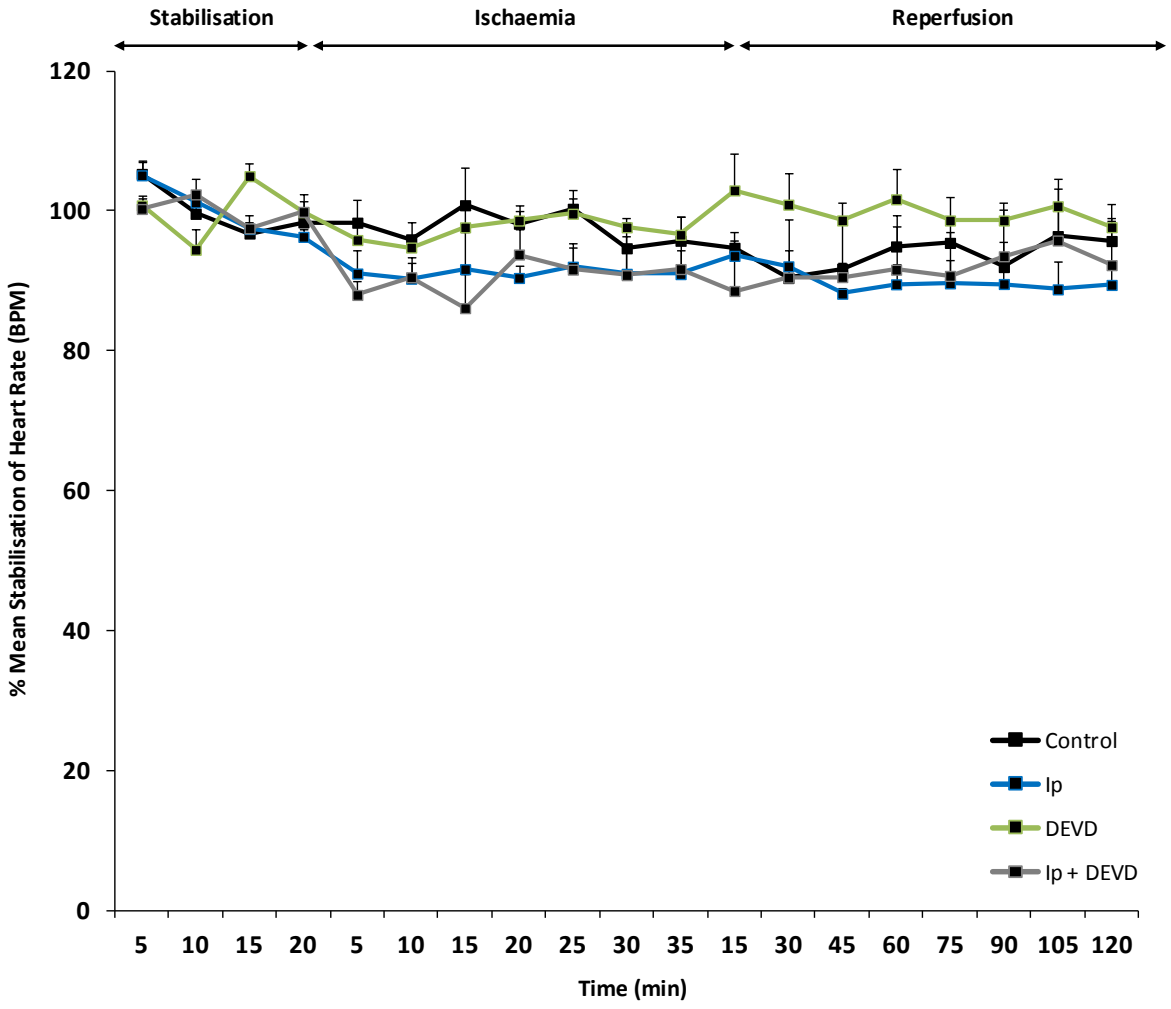


Figure 3.31: Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1 x 10^{-7} M) ± DEVD (7 x 10^{-8} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.3.12.5 Coronary flow (CF)

There was no significant difference in CF in comparison with the control with hearts subjected to ipratropium (1 x 10^{-7} M) or DEVD (7 x 10^{-8} M) ± ipratropium (1 x 10^{-7} M) administration at any time point, for any of the experimental groups (Figure 3.32).

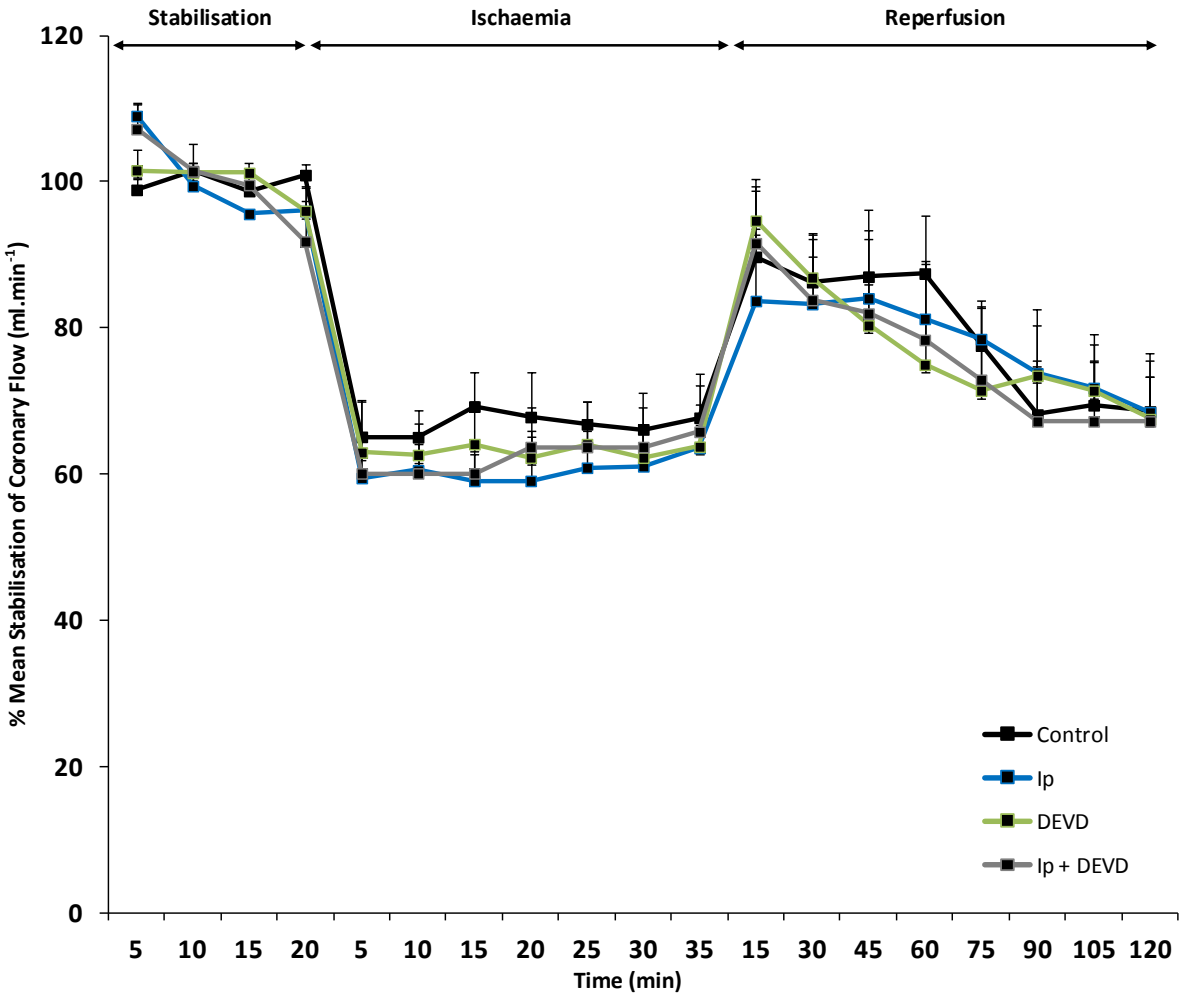


Figure 3.32: Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1 x 10^{-7} M) ± DEVD (7 x 10^{-8} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

3.4 Chapter Discussion

The identification that ipratropium induced injury did not occur under normoxic conditions is of particular importance as it indicates that the action of ipratropium on specific signalling pathways activated during ischaemia, reperfusion, or both is responsible for the increase in myocardial injury. In particular, it appears that the observed ipratropium induced effect is due to ipratropium induced alteration of reperfusion signalling, as when ipratropium was administered at different time points throughout the Langendorff protocol, the observed increase in infarction was statistically insignificant across the different time points.

The major finding of this work is that ipratropium significantly exacerbates myocardial injury under *in vitro* conditions of simulated myocardial I/R and oxidative stress. This was observed as an increase in infarct size (I/R%) in the isolated perfused rat heart and as a decrease in myocyte viability in primary rat ventricular myocytes, established via a decrease in MTT reductase activity. The present study also demonstrates the ability of acetylcholine to abrogate the exacerbation of I/R injury in these two models. This accords with previous studies that have shown muscarinic activation is cytoprotective following an ischaemic insult (Davidson et al. 2006, Qin, Downey and Cohen 2003, Zhao et al. 2010). Interestingly, in the current study, acetylcholine was capable of attenuating some of the observed ipratropium induced injury when ipratropium and acetylcholine were co-administered. This indicates that the inhibitory action of ipratropium, as a competitive antagonist, at the cardiac mAChRs is responsible for eliciting the observed injury.

In the current study, the presence of acetylcholine in *in vitro* tissue was confirmed. It has been approximately three decades since the interest in synthesis of ACh in non-neuronal cells became a particular focus for research (Grando et al. 2007). It has since been shown that ACh is actually synthesised, *in vivo*, by the majority of non-neuronal cells and plays integral roles in various non-neuronal capacities (Wessler and Kirkpatrick 2008).

It was initially shown by Dolezal and Tucek that ACh synthesis occurs *ex vivo* in the diaphragm of the rat (Tucek 1982). It has since been demonstrated that ChAT mRNA, as well as ChAT protein, are active in isolated human epithelial and lymphocytes (Grando et al. 1993, Kawashima and Fujii 2004) as well as ChAT activity and ACh synthesis in the airway epithelium and placenta (Grando et al. 1993, Reinheimer et al. 1996, Wessler et al. 2001).

For the purposes of the work in the current study, it was necessary to identify endogenous ACh levels within the *in vitro* models utilised, given that it was postulated that it was a specific action on the mAChRs by ipratropium, which was eliciting the cardio-toxic effects. Concentrations of 1.3 – 1.7 x 10^{-9} M were identified in the myocyte lysates. This is in-line with previous work which indicates endogenous, non-neuronal, ACh levels range from 9 x 10^{-12} M and 6 x 10^{-8} M (Wessler and Kirpatrick, 2008).

Further to this, ipratropium administration was sufficient to significantly increase myocardial injury in the absence of exogenous acetylcholine (1 × 10^{-7} M). Previous work has shown that acetylcholine protects the myocardium following I/R (Li et al. 2011) by permitting activation of reperfusion injury salvage kinase (RISK) signalling cascades (Hausenloy, Tsang and Yellon 2005, Shanmuganathan et al. 2005),

maintenance of gap junctional integrity (Yue et al. 2006, Zhao et al. 2010), and prevention of apoptosis (Kim et al. 2008a, Liu et al. 2011a). This study supports the protective properties of acetylcholine and postulates that the administration of ipratropium is sufficient to compete with endogenous acetylcholine, which accounts for the observed inhibition of acetylcholine induced myocardial protection following I/R.

The parallel studies using atropine have generated supporting evidence for muscarinic involvement as, through this work, increases in infarct development and cleaved capase-3 levels as well as decreased myocyte viability were observed with atropine administration. It was also demonstrated that acetylcholine was capable of compensating for some of the observed atropine induced injury when administered in conjunction with atropine. Ipratropium is a synthetic atropine derivative (Wood et al. 1995), and both drugs are non-selective muscarinic antagonists. The ability for both atropine and ipratropium to exacerbate myocardial injury in the experimental models presented here implies a common mechanism of action of the two drugs which reinforces the theory that muscarinic signalling may be involved in ipratropium-induced exacerbation of myocardial injury. During reperfusion, myocyte loss can occur through both apoptosis and necrosis (Kung, Konstantinidis and Kitsis 2011). Administration of ipratropium was associated with an increase in both necrotic and apoptotic myocyte death in comparison with the untreated group, as well as an overall decrease in viable myocytes.

Furthermore, it was demonstrated that ipratropium administration, at the onset of re-oxygenation in isolated rat cardiac myocytes, was associated with an increase in levels

of cleaved caspase-3. Caspase-3 is an essential effector caspase in the caspase-dependent apoptotic signalling cascade. Caspases exist as zymogens but are only cleaved to form active enzymes following the onset of apoptosis. Significant increases in cleaved caspase-3 levels were observed following ipratropium administration, thereby indicating that ipratropium exacerbation of myocardial injury involves a caspase-dependent apoptotic component. This was further supported by the isolated perfused heart studies using ipratropium and Z-DEVD-FMK (DEVD), an irreversible inhibitor of caspase-3 activation. These data identified that the observed ipratropium-induced increase in I/R (%) was abrogated when administered in conjunction with DEVD. The levels of caspase-3 in the acetylcholine treatment group were significantly lower than in the control group, indicating that acetylcholine myocardial protection is, in part, due to a reduction in apoptosis. This is also supported by other studies where acetylcholine-induced cardioprotection has been shown to protect via regulation of apoptosis (Kim et al. 2008a, Liu et al. 2011a) and promotes the hypothesis that caspase activation is necessary in order for ipratropium to exacerbate myocardial injury following I/R. However, from other results in this study, it is implied that there is also a necrotic component to the observed ipratropium induced myocyte loss in an *in vitro* setting of I/R.

Chapter 4 Elucidation of the cellular signalling pathways which lead to ipratropium induced myocardial injury

4.1 Chapter introduction and purpose

In the previous chapter (Chapter 3), it was demonstrated that ipratropium is capable of exacerbating myocardial injury following simulated ischaemia/reperfusion (I/R) in the isolated Langendorff perfused rat heart model as well as primary rat ventricular myocytes. This was identified as a significant increase in both apoptosis and necrosis following ipratropium administration, coupled with an overall decrease in viability of cardiac myocytes. The presence of endogenous acetylcholine (ACh) was ascertained, however the administration of exogenous acetylcholine was capable of partially reversing the ipratropium induced injury, implying that it is the specific antagonist action of ipratropium at cardiac muscarinic receptors that is responsible for the observed increase in injury. The cytoprotective properties of muscarinic activation, by ACh, have been well documented. In the context of I/R, ACh has been shown as cardio-protective (Kim et al. 2008a, Li et al. 2011, Yang et al. 2005) as well as effective in both pre-conditioning (Critz, Cohen and Downey 2005, Krieg et al. 2004, Yang et al. 2005) and post-conditioning (Xiong et al. 2010, Zang, Sun and Yu 2007).

Mechanistically, ACh mediated cardio-protection has also been shown to inhibit hypoxia-induced apoptosis through regulation of MAPKs, Akt and PI3K (Kim et al. 2008a, Krieg et al. 2002, Li et al. 2011), as well as Bcl-2 family proteins and caspase-3 (Kim et al. 2008a, Yao et al. 1999). The work presented in the previous chapter

supports this as administration of acetylcholine induced a reduction in apoptosis which was accompanied by decreases in caspase-3 levels. However, this effect was ameliorated following co-administration of ACh and ipratropium. It has previously been demonstrated that synthesis of ACh, in the myocardium, is increased following conditions of I/R in feline (Kawada et al. 2007) and rabbit models (Kawada et al. 2009). In particular, ACh levels rapidly increase during ischaemia, and remain elevated throughout reperfusion (Kumagae and Matsui 1991). This provides an innate protection to myocytes following an ischaemic event (Kawada et al. 2009). It is therefore postulated that ipratropium is capable of blocking endogenous ACh at the cardiac muscarinic receptors, therefore blocking cardio-protective cholinergic mechanisms.

The reperfusion injury salvage kinase (RISK) pathway comprises of PI3K-Akt and, the mitogen-activated protein kinases (MAPKs), p42/p44, extracellular signal-regulated kinases (Erk1/2) pathways (Davidson et al. 2006). Its activation at the onset of reperfusion is capable of decreasing the severity of reperfusion injury (Hausenloy, Tsang and Yellon 2005). Previous work has shown that ACh is capable of protecting the myocardium following I/R by permitting activation of pivotal kinases in the RISK pathway (Li et al. 2011). More specifically, ACh has been shown as protective against I/R when used in both pre- and post-conditioning due to its ability to activate the RISK pathway (Li et al. 2011, Mioni et al. 2005, Yang et al. 2005, Zhao et al. 2010).

MAPKs are serine and threonine kinases which are also instrumental in regulating a myriad of cellular processes including cellular survival and apoptosis (Chen et al. 2001). The MAPK JNK is stress activated and known to play an integral role in the initiation of

apoptosis in various disease states, including I/R, following its phosphorylation and subsequent activation (Zhao and Herdegen 2009). The activation of JNK includes both intrinsic and extrinsic apoptotic pathways, whereas inhibition of JNK permits the attenuation of apoptosis in several cell types and under various cellular contexts (Son, Rhee and Pyo 2006, Venugopal et al. 2007). The activation of muscarinic receptors, in particular the M_3 subtype, following innervation by endogenous ACh, has also been shown to inhibit JNK activation (Kim et al. 2008a, Wang et al. 2003). Thereby providing evidence for another potential mechanism by which ACh employs cardio-protective signalling pathways.

During I/R, increases of intracellular Ca^{2+} and oxidative stress lead to rapid ATP depletion, causing rupture of the outer mitochondrial membrane and, thus, MOMP. This facilitates the influx of toxic mitochondrial proteins into the cytosol, consequently initiating apoptosis (Halestrap and Pasdois 2009, Hausenloy et al. 2002) as well as myocyte death by necrosis (Yellon and Hausenloy 2007b) which occurs via the resultant collapse of mitochondrial function after disruption of mitochondrial membrane potential (Crompton 1999) due to opening of the mPTP (Yellon and Hausenloy 2007b). In contrast to MOMP, mPTP opening is primarily responsible for myocyte loss through necrosis, rather than apoptosis, as attributed to the former. The two were previously considered as distinct components responsible for separate cell death mechanisms (Siu et al. 2008), however, it has recently emerged that Bax/Bak channels may be integral for mPTP functioning (Whelan et al. 2012).

Irrespective of the mechanisms involved, ACh has shown roles in protecting the myocardium from I/R injury via regulation of the MOMP, through modulation of Bak

and Bax (Liu et al. 2011a) as well as prevention of mPTP opening whereby ACh induced cardioprotection has been shown to be abolished by atractyloside, a known opener of the mPTP (Sun et al. 2010a). This indicates an integral role for both the MOMP and mPTP in acetylcholine mediated cardio-protection.

Due to the convergence of muscarinic and RISK signalling, as well as the association with the mPTP, it is therefore possible that the antagonist action of ipratropium on mAChRs in the heart, and the observed exacerbation of myocardial injury, may involve PI3K-Akt and Erk1/2 signalling. The aim of the following work was to attempt to elucidate whether the pro-apoptotic Bcl-2 family member BAD (to further define the involvement of apoptosis), Akt and Erk1/2 (components of both the RISK and muscarinic signalling cascades), JNK (an integral component in the apoptotic machinery following cellular stresses) and mitochondrial membrane potential and rigor contracture of cardiac myocytes (following oxidative stress, which indicate involvement of the mPTP) are involved in the cellular mechanisms which lead to ipratropium induced injury.

4.2 Methods

70 adult, male, Sprague-Dawley rats (300 g ± 50 g body weight) were used throughout the experiments detailed in this chapter. All experiments were subject to the exclusion criteria detailed in Chapter 2, section 2.8. For all data, results are presented a group means + standard error of the mean (SEM). For the Western blotting data, values have been normalised to the respective untreated control, which is represented as 100%. Statistical differences were ascertained with the use of one-way ANOVA, with the exception of haemodynamics which where a two-way ANOVA was employed, and Fisher's LSD post-hoc test.

4.2.1 mPTP model of oxidative stress

Cardiac myocytes were isolated according to the protocol in Chapter 2, section 2.4. Following adhesion to laminin coated cover slips, adhered myocytes were incubated in microscopy buffer containing 3 x 10^{-6} M, tetramethylrhodamine methyl ester (TMRM) for 15 minutes. Cells were washed and randomly assigned to one of the following groups: untreated control, Ip (1 x 10^{-8} M - (1 x 10^{-6} M),), ACh (1 x 10^{-7} M), or Ip + ACh (both 1 x 10^{-7} M). Myocytes were viewed and analysed via confocal microscopy. Depolarisation (Dep) was measured as the time at which the TMRM started to become evenly distributed throughout the cell and is indicative of the initiation of the mPTP opening. Subsequent hypercontracture (Hyp) of myocytes occurs shortly afterwards due to ATP depletion. The time to both Dep and Hyp were recorded.

4.2.2 Western blotting

Tissue was harvested from Langendorff perfused rat hearts following 20 minutes stabilisation, 35 minutes regional ischaemia and then 5, 15 or 120 minutes reperfusion, as described in Chapter 2, section 2.6. All drugs were administered at the onset of, and throughout, reperfusion, for this study, these were ipratropium (1×10^{-9} M – 1×10^{-7} M), ACh (1×10^{-7} M) and wortmannin (1×10^{-8} M), as well as ipratropium (1×10^{-7} M) administered concomitantly with either ACh or wortmannin (concentrations as before). For groups where drugs were co-administered, 1×10^{-7} M of ipratropium was used. Following homogenisation and storage in suspension buffer, 60 µg of protein from each sample was diluted with sample buffer and loaded into precast gels. After separation by electrophoresis, proteins were transferred onto PVDF membranes and probed for the phosphorylated forms of Akt (Ser_{473}, 60 KDa), Erk 1/2 (Erk) (Thr_{202}/Thr_{204}, 44/42 KDa) and SAPK/JNK (JNK) (Thr_{183}/Tyr_{185}, 54/46 KDa). To assess relative changes in protein levels, membranes were subsequently probed for the total, unphosphorylated forms of Akt, Erk and JNK and relative changes in phosphorylated protein were calculated as a percentage of these. To ensure protein loading was equal, all membranes were also probed for GAPDH.

4.2.3 Analysis of adult rat ventricular myocytes via flow cytometry

Isolated cardiac myocytes were subjected to the H/R protocol, as detailed in Chapter 2, section 2.4.1. Ipratropium (1×10^{-9} M – 1×10^{-6} M), atropine (1×10^{-7} M) and ACh (1×10^{-7} M), as well as ipratropium (1 x 10-7 M) in conjunction with either atropine or ACh (concentrations as before) were administered for four hours, following two hours hypoxia. At the end of the experimental protocol, levels of phospho-Akt (Ser_{473}) and phospho-BAD (Ser_{112}) were assessed by flow cytometry (protocol outlined in Chapter 2, section 2.4.6).

4.2.4 Isolated perfused rat heart model of ischaemia/reperfusion

Following sacrifice by cervical dislocation and excision, rat hearts were mounted on Langendorff perfusion apparatus. For all experiments, the Langendorff model of I/R was used. Ipratropium (1×10^{-7} M) ± Wortmannin (1×10^{-8} M) and Wortmannin alone (1×10^{-8} M) were administered after 55 mins, the onset of reperfusion. At the end of the experimental protocol, infarct size to risk ratio (I/R(%)) was determined via Evans blue and TTC staining. Throughout all experiments, left ventricular developed pressure (LVDP, mmHg), heart rate (HR, $beats.min^{-1}$) and coronary flow (CF, $ml.min^{-1}$) were measured to assess the stability of the hearts.

4.3 Results

4.3.1 Involvement of mitochondria following ipratropium and acetylcholine administration in a mPTP model of oxidative stress

Having identified involvement of both apoptotic and necrotic myocyte death following ipratropium administration in Chapter 3, a myocyte model of oxidative stress was employed to determine the action of ipratropium on mitochondria. Figure 4.1 (**A**) portrays the time points which were visualised and measured (depolarisation and hypercontracture) via confocal microscopy. The administration of ipratropium (1×10^{-8} M and 1×10^{-7} M) significantly decreased the time taken for depolarisation to occur in comparison with the control group (201.2 ± 9.9 s, control vs. 173.8 ± 14.6 s (1×10^{-8} M) and 147.5 ± 17.5 s (1×10^{-7} M), $p<0.05$, Figure 4.1 (**B**)). The subsequent time to hypercontracture was also recorded, as presented in Figure 4.1 (**C**). As with depolarisation, there was a significant decrease in hypercontracture following ipratropium (1×10^{-8} M and 1×10^{-7} M) administration (585.3 ± 33.9 s, control vs. 460.5 ± 19.0 s (1×10^{-8} M) and 455.2 ± 15.3 s (1×10^{-7} M), $p<0.05$). In order to determine whether ACh elicited protection via a mechanism involving mitochondria, the same protocol in the presence of ACh ± ipratropium was conducted. ACh administration, alone (277.2 ± 26.1 s, $p<0.01$), and in conjunction with ipratropium (237.0 ± 9.4 s, $p<0.05$), was capable of significantly delaying time to depolarisation in comparison with the control (Figure 4.1 (**D**)). The subsequent time to hypercontracture was also recorded (Figure 4.1 (**E**)). These results mirrored those from depolarisation (455.1 ± 15.2 s, Ip, $p<0.01$, 572.9 ± 23.4 s, ACh, $p<0.05$ and 547.7 ± 12.1 s, Ip + ACh vs. 550.2 ±

1.7 s in control), with the exception that statistically there was no difference between the control and Ip + ACh treated groups.

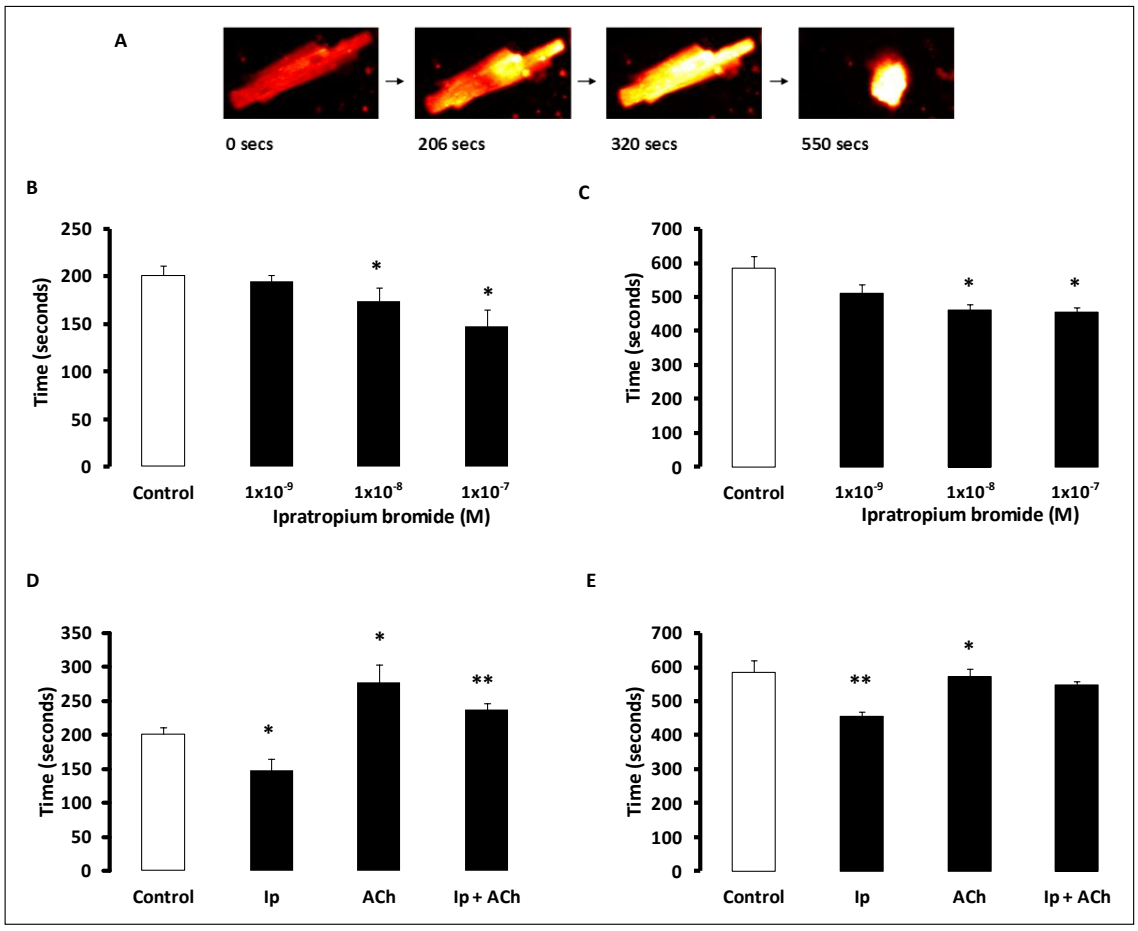


Figure 4.1 Determination of myocyte depolarisation and hypercontracture as established using TMRM loaded cells subjected to laser stimulated oxidative stress generation. Time points measured are schematically represented (**A**) the time to depolarisation, 2nd panel, (201.2 ± 9.9 secs) and hypercontracture, 4th panel (585.3 ± 33.9 secs), as visualised by confocal microscopy. Effect of ipratropium (1×10^{-9} M – 1×10^{-7} M) on time to depolarisation (**B**) and hypercontracture (**C**). Effect of acetylcholine (ACh, 1×10^{-7} M) ± ipratropium (Ip, 1×10^{-7} M) on time to depolarisation and hypercontracture (**D** and **E**, respectively), values are mean + SEM, n=6 animals, with between 10 and 15 myocytes measured, per animal, for each group. *p<0.05, ** p<0.01 vs. respective control (white bars).

4.3.2 Effect of ipratropium treatment on the levels of Akt phosphorylation following ischaemia/reperfusion

As Akt is an integral protein in the RISK signalling machinery and also involved in muscarinic signalling, the levels of phospho- and total-Akt following 35 minutes ischaemia and 5, 15 and 120 minutes of reperfusion were quantified via Western blot analysis. Administration of all drugs (ipratropium (1×10^{-9} M – 1×10^{-7} M), ACh (1×10^{-7} M), wortmannin (1×10^{-8} M) and ipratropium (1×10^{-7} M) administered in conjunction with either ACh or wortmannin (concentrations as before) occurred at the onset of, and throughout, reperfusion.

4.3.2.1 Ipratropium induced myocardial injury is associated with an up-regulation of phospho-Akt

Figure 4.2 presents the data from Western blot analysis where isolated perfused hearts were exposed to ipratropium (1×10^{-9} – 1×10^{-7} M) throughout reperfusion (which lasted for 5 (**A**), 15 (**B**) and 120 (**C**) minutes. A significant increase in levels of phospho-Akt was observed following administration of ipratropium (all concentrations) at all time points, as described in Fig. 4.2 (**D**) ($p<0.01$ vs. respective untreated control for all groups).

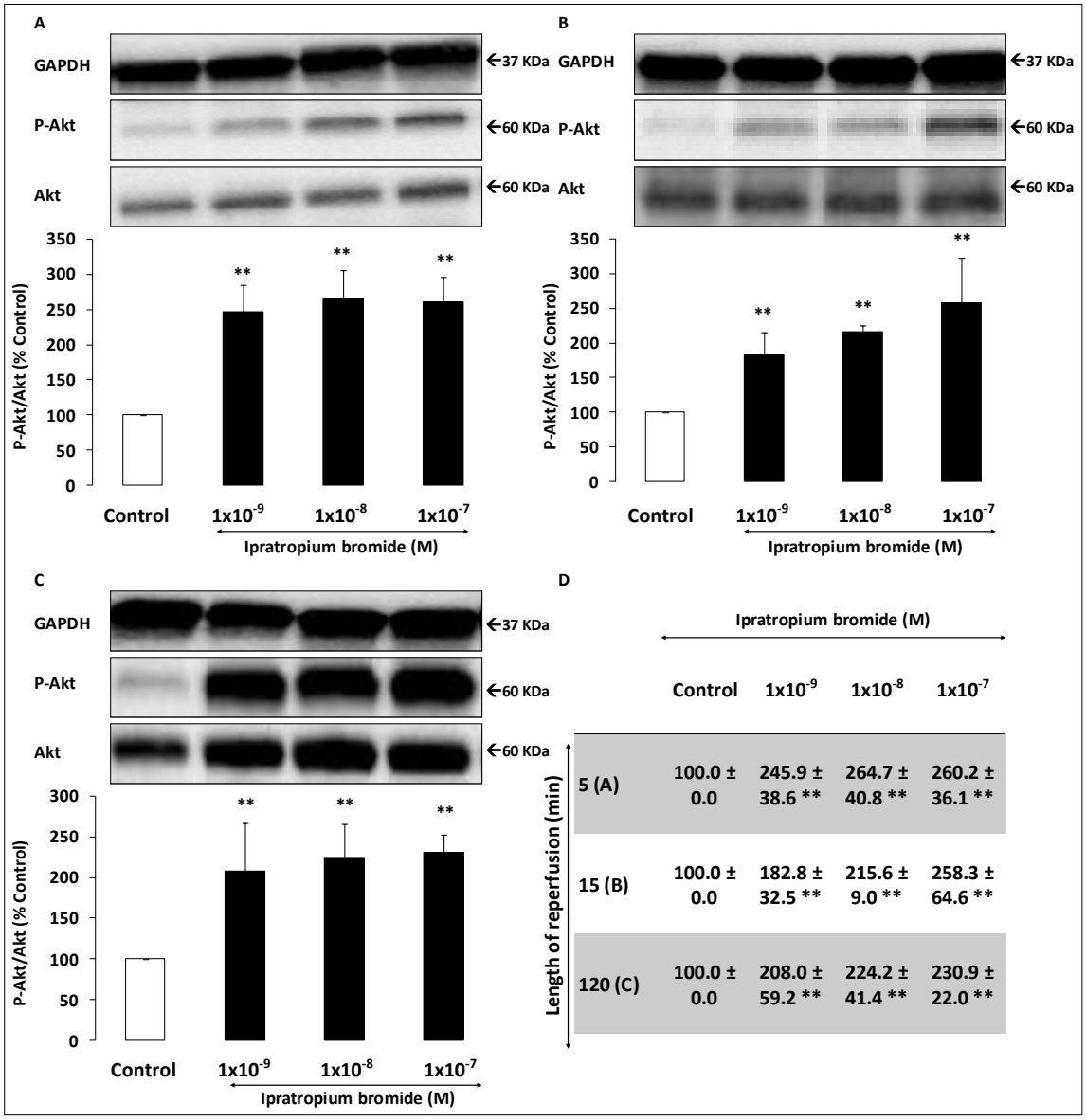


Figure 4.2: The effects of ipratropium (1 x 10^{-9} M – 1 x 10^{-7} M) treatment on the levels of phosphorylated Akt following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-Akt and total Akt for each time point shown above figures with treatment groups in the following order for all blots: Control, Ip (1 x 10^{-9} M), Ip (1 x 10^{-8} M), and Ip (1 x 10^{-7} M),. All values expressed as mean + SEM of p-Akt/Akt as a percentage of the control (**D**). * p<0.05 and ** p<0.01 vs. respective control, n=3 for all groups.

4.3.2.2 Acetylcholine mediated protection against ipratropium induced myocardial injury is associated with downregulation of phospho-Akt

Figure 4.3 presents the results from the Western blot experiments designed to ascertain levels of Akt following ipratropium ± ACh. Administration of ACh (1×10^{-7} M) produced an effect which was statistically indifferent from the respective control groups at all time points measured. However, at all time points there was a significant decrease in Akt phosphorylation in the groups treated with ACh in comparison with the respective ipratropium treated group (Figure 4.3 (**D**), $p<0.05$ for all groups). The co-administration of ipratropium and ACh, following 5 minutes reperfusion, significantly reduced Akt phosphorylation in comparison with the group treated with ipratropium alone (260.2 ± 36.1%, Ip, vs. 178.7 ± 23.2%, Ip + ACh, $p<0.05$). The relative change in Akt phosphorylation following 15 minutes reperfusion was also statistically different between the ipratropium and Ip + ACh treatment groups (258.3 ± 64.6%, Ip, vs. 127.6 ± 28.7%, Ip + ACh, $p<0.05$), however, the difference between the same two respective groups (Ip compared with Ip + ACh) following 120 minutes reperfusion was insignificant (Figure 4.3 (**C**)).

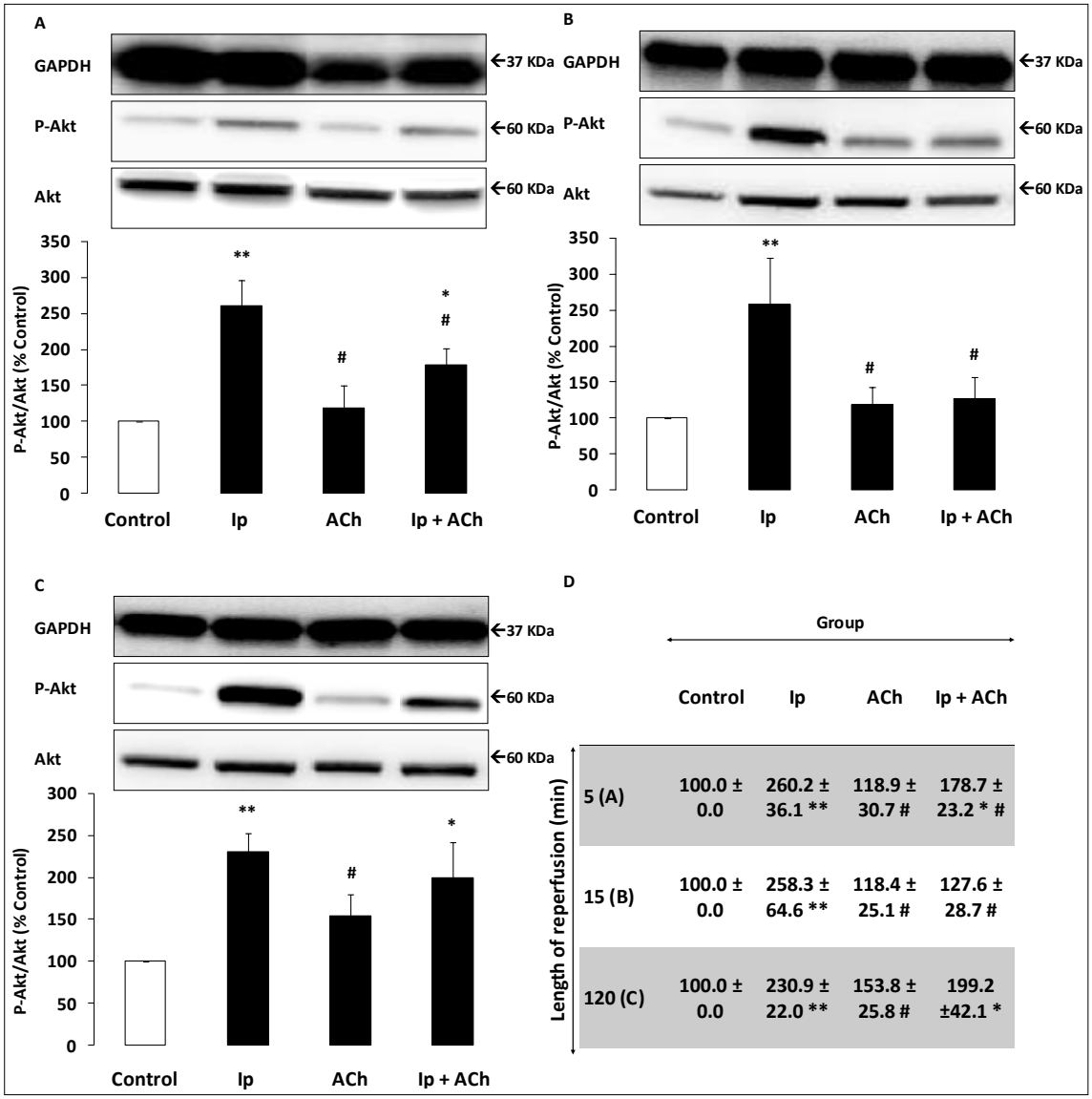
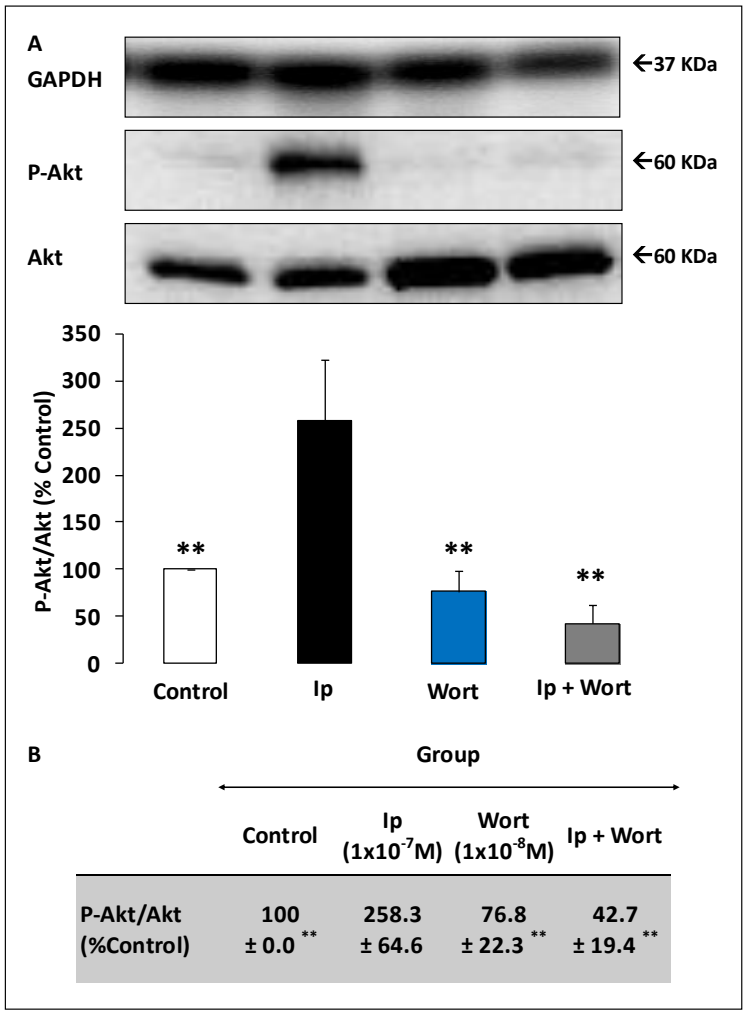


Figure 4.3: The effects of ipratropium (1 x 10^{-7} M) ± acetylcholine (1 x 10^{-7} M) treatment on the levels of phosphorylated Akt following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-Akt and total Akt for each time point shown above figures with treatment groups in the following order for all blots: Control, Ip, ACh and Ip + ACh. All values expressed as mean + SEM of p-Akt/Akt as a percentage of the control (**D**). * $p<0.05$ and ** $p<0.01$ vs. respective control, # $p<0.05$ vs. respective Ip treated group, n=3 for all groups.

4.3.2.3 Western blotting – AKT ipratropium ± wortmannin study

In order to ascertain whether the observed increases in Akt phosphorylation following ipratropium administration involved the PI3K, wortmannin, a selective PI3K inhibitor, was administered at reperfusion in the presence and absence of ipratropium (Figure 4.4). Administration of wortmannin (1 x 10^{-8} M) blocked Akt phosphorylation when administered alone and in conjunction with ipratropium (1 x 10^{-7} M) (Figure 4.4 (**B**).



	Control	Ip ($1x10^{-7}$M)	Wort ($1x10^{-8}$M)	Ip + Wort
P-Akt/Akt (%Control)	100 ± 0.0 **	258.3 ± 64.6	76.8 ± 22.3 **	42.7 ± 19.4 **

Figure 4.4 (**A**) The effects of ipratropium (1 x 10^{-7} M) ± wortmannin (1 x 10^{-8} M) treatment on the levels of phosphorylated Akt following 35 minutes regional ischaemia 15 minutes reperfusion. Corresponding representative blots of GAPDH, p-Akt and total Akt shown above figures with treatment groups in the following order for all blots: Control, Ip, Wort and Ip + Wort. All values expressed as mean + SEM of p-Akt/Akt as a percentage of the control (**B**). ** $p<0.01$ vs. Ipratropium (Ip, 1 x 10^{-7} M), n=3 for all groups.

4.3.2.4 Isolated Perfused Rat Heart Experiments with ipratropium ± wortmannin Treatment

Following the identification that wortmannin was capable of limiting ipratropium induced Akt phosphorylation, perfused Langendorff rat heart experiments were carried out using ipratropium ± wortmannin. Interestingly, as presented in Figure 4.5, co-administration of ipratropium and wortmannin was incapable of limiting the observed increase in infarct development due to ipratropium administration.

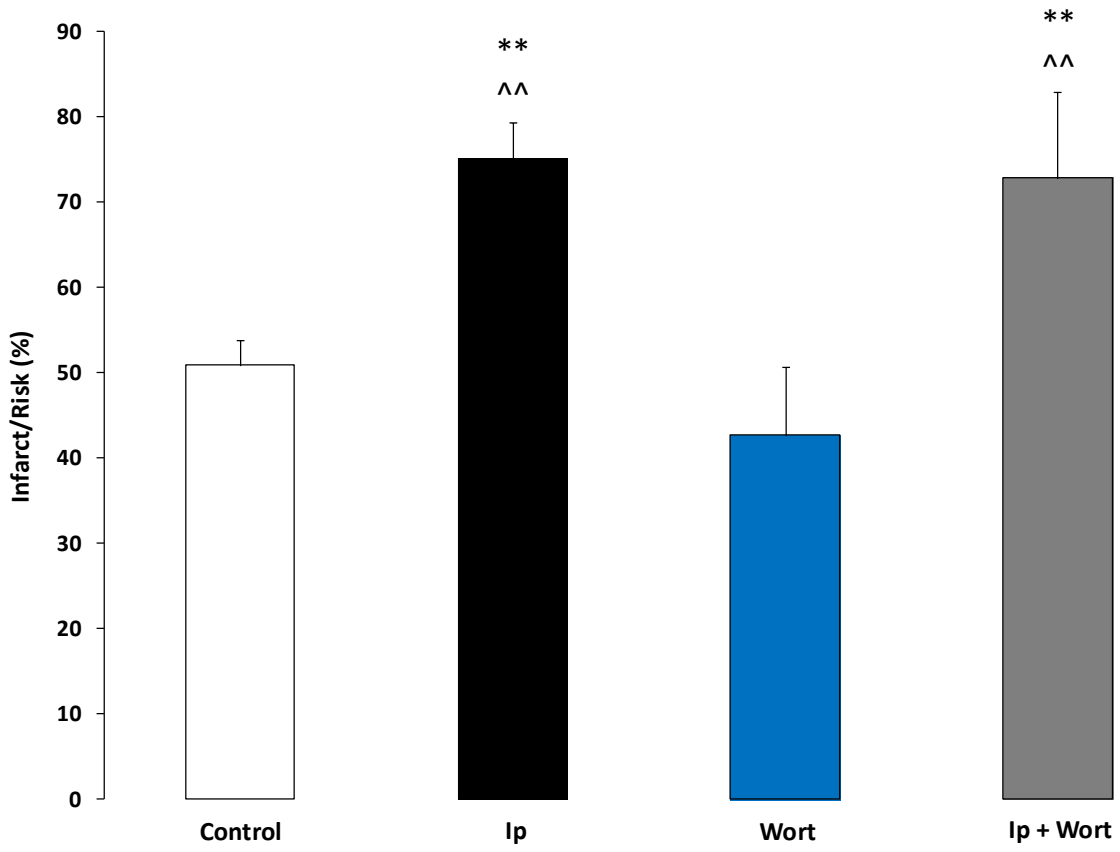


Figure 4.5: Infarct development in the risk zone following wortmannin (Wort, 1 x 10^{-8} M) treatment ± ipratropium (Ip, 1 x 10^{-7} M) in isolated perfused rat heart. Wortmannin and ipratropium both administered at the onset of reperfusion. Results are presented as infarct/risk (%). **p<0.01 vs. Control, ^^p<0.01 vs. wortmannin (Wort, 1 x 10^{-8} M). Data presented as arithmetic mean + SEM, n=6.

4.3.2.5 Haemodynamic data analysis

The haemodynamic parameters of left ventricular developed pressure, heart rate and coronary flow were measured at regular intervals throughout perfusion on the Langendorff apparatus. All haemodynamic parameters were measured and expressed as previously described (Chapter 2, section 2.3.3) and are presented in Figures 4.6 (LVDP), 4.7 (HR) and 4.8 (CF). There were no significant differences in LVDP, HR or CF between the control, or any of the ipratropium ± wortmannin treatment groups at any time point.

4.3.2.5.1 Left Ventricular developed Pressure

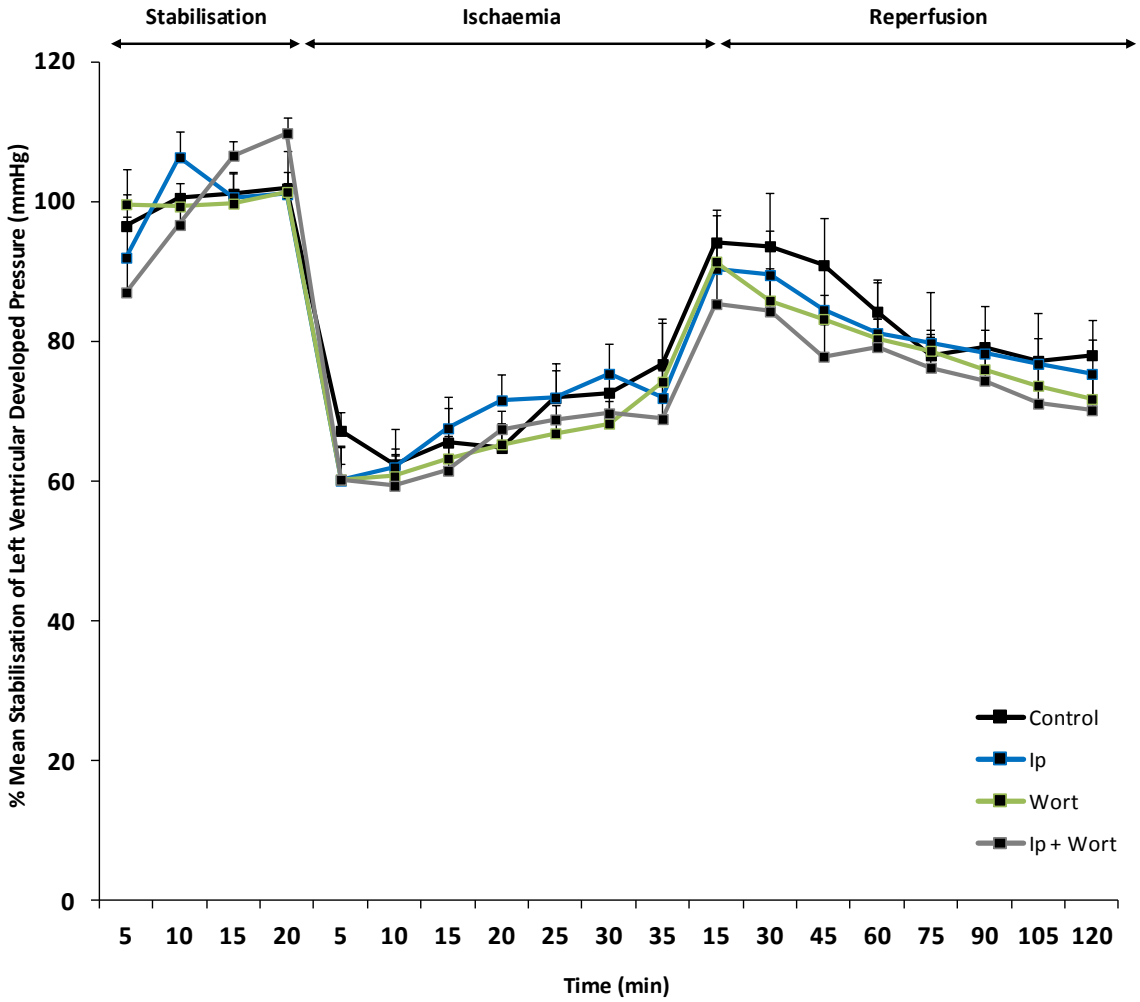


Figure 4.6: Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1×10^{-7} M) ± wortmannin (1×10^{-8} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

4.3.2.5.2 Heart Rate

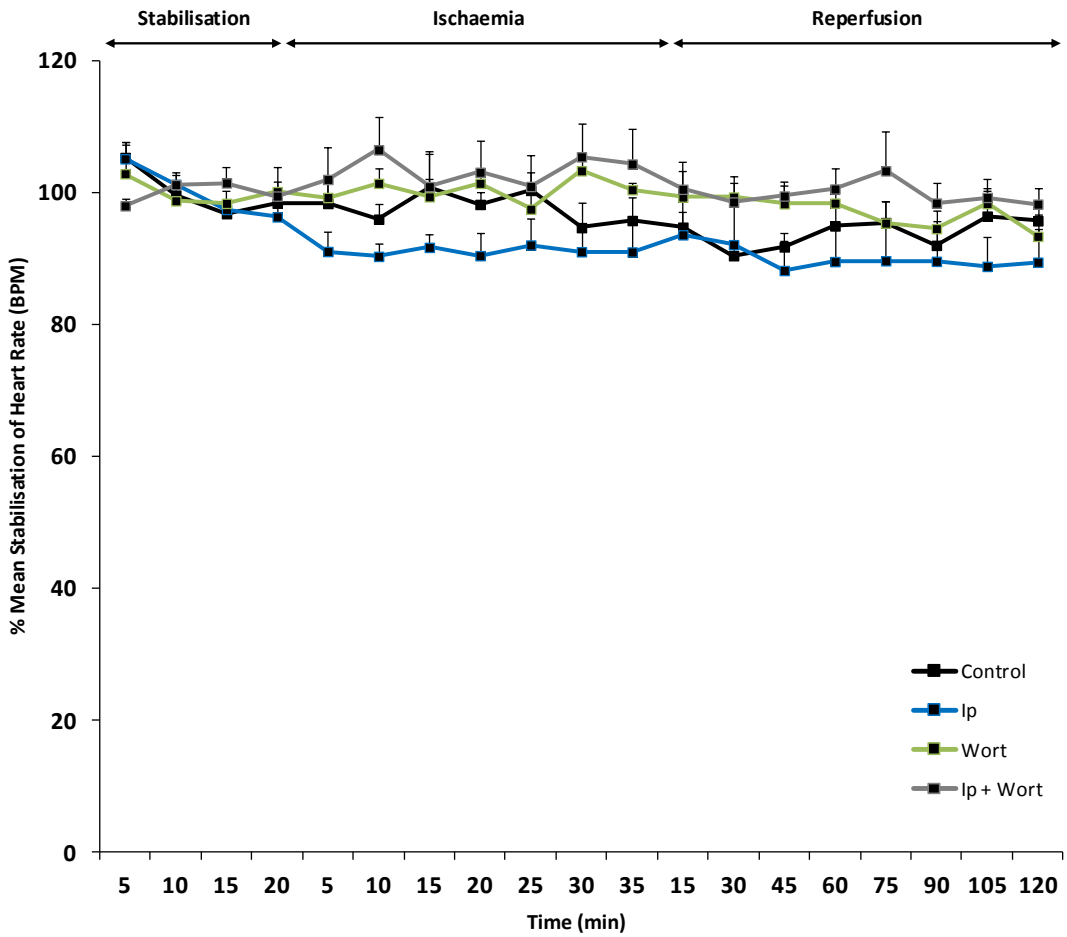


Figure 4.7: Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1×10^{-7} M) ± wortmannin (1×10^{-8} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

4.3.2.5.3 Coronary Flow

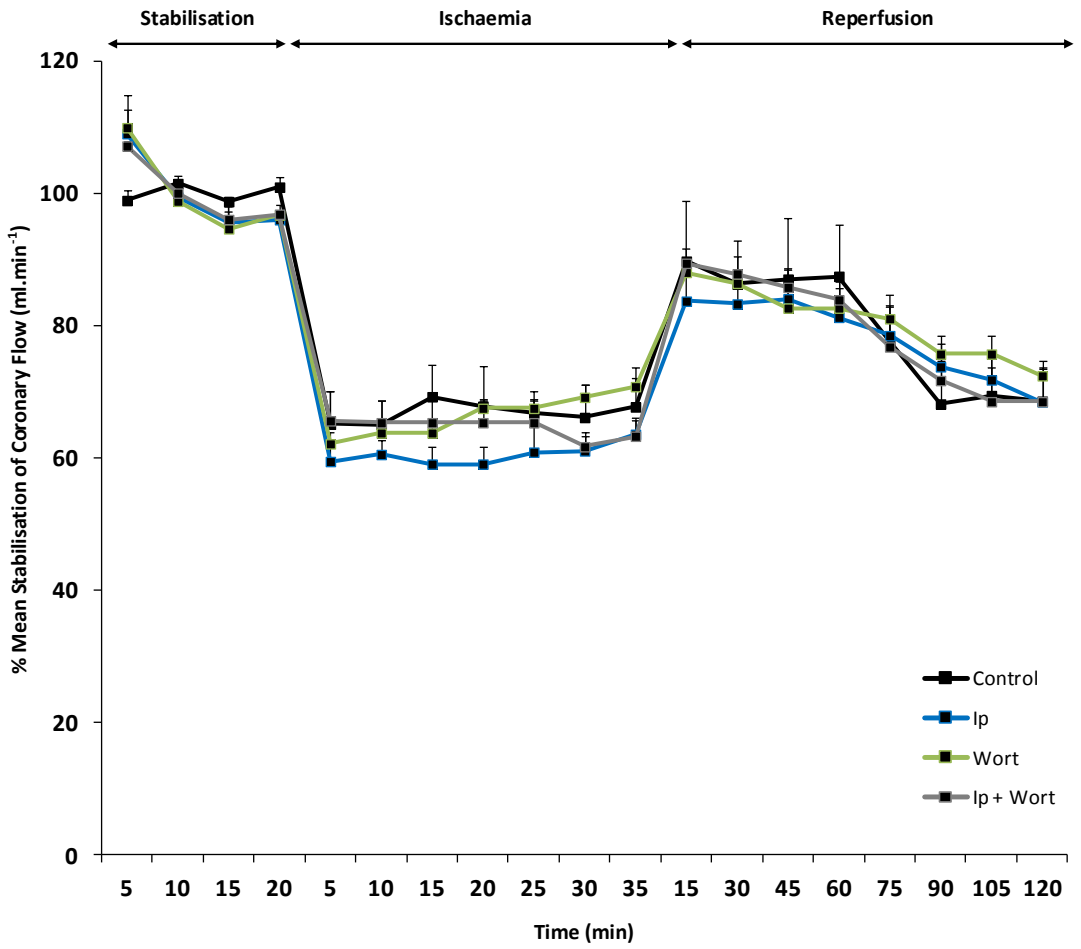


Figure 4.8: Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium bromide (1 x 10^{-7} M) ± wortmannin (1 x 10^{-8} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups, $p > 0.05$.

4.3.3 Effect of ipratrpoium treatment on the levels of Erk1/2 phosphorylation following ischaemia/reperfusion

In order to ascertain whether Erk1/2 expression is involved in ipratropium induced myocardial injury, hearts were harvested for Western blot analysis as previously described, following 5, 15 and 120 minutes reperfusion where ipratropium ± ACh were administered at the onset of and throughout reperfusion.

4.3.3.1 Ipratropium induced myocardial injury is associated with an up-regulation of Erk1/2 phosphorylation

Figure 4.9 presents the data from this study. An increase in levels of phospho-Erk1/2 was observed at all time points following ipratropium (1×10^{-9} M – 1×10^{-7} M) administration in comparison with the respective untreated control groups. These observed increases were significant in comparison with the control groups ($p<0.05$ or $p<0.01$, values in Figure 4.9 (**D**)) with the exception of the 1×10^{-9} M treatment group following 15 minutes of reperfusion (111.9 ± 9.1%, Ip vs. 100.0 ± 0.0%,control, not significant).

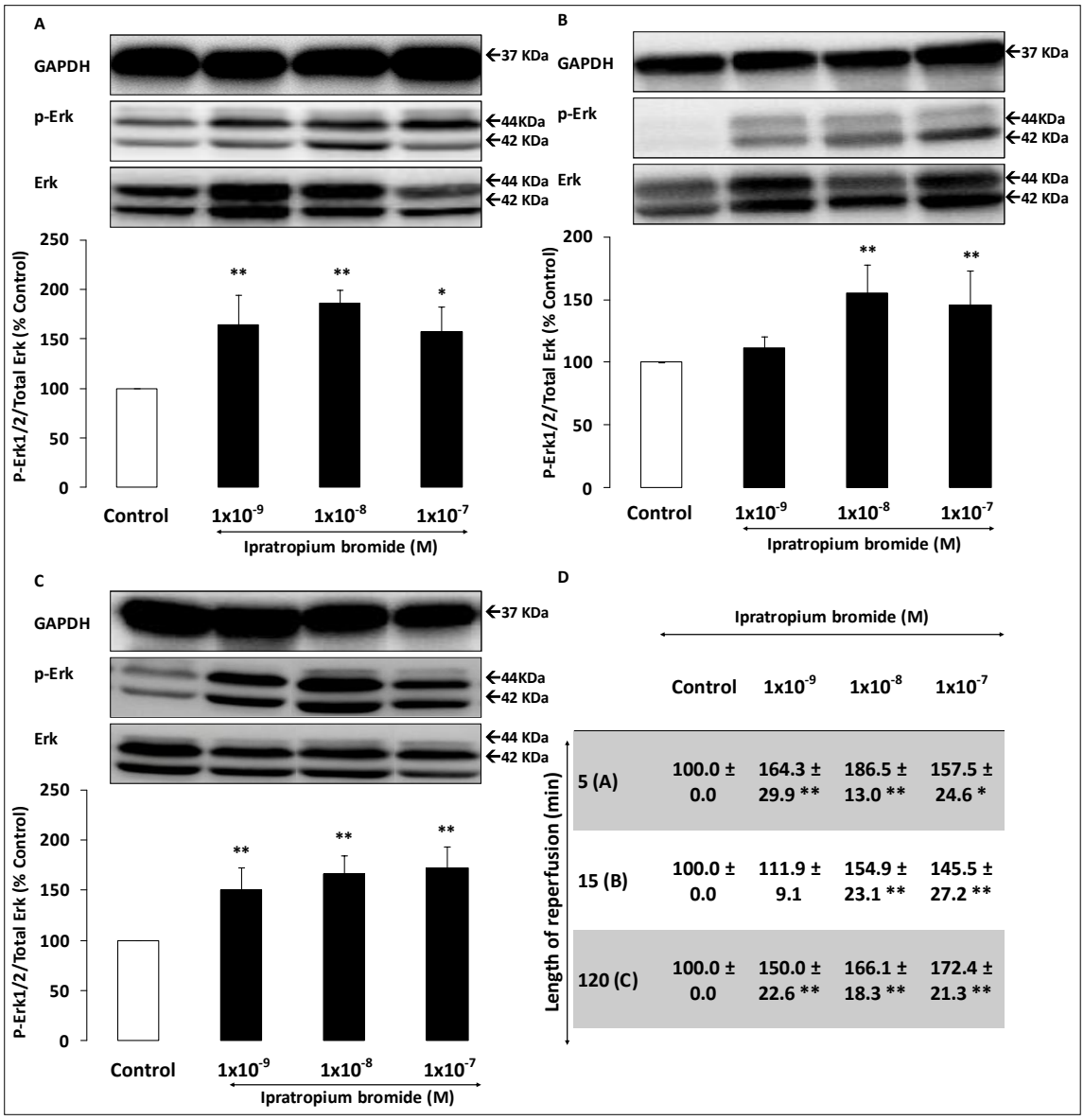


Figure 4.9 The effects of ipratropium (1 x 10^{-9} M – 1 x 10^{-7} M) treatment on the levels of phosphorylated Erk1/2 following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-Erk1/2 and total Erk1/2 for each time point shown in the above figures. Treatment groups are presented in the following order for all blots: Control, Ip (1 x 10^{-9} M), Ip (1 x 10^{-8} M), and Ip (1 x 10^{-7} M),. All values expressed as mean + SEM of p-Erk1/2/Erk1/2 as a percentage of the control (**D**). * $p<0.05$ and ** $p<0.01$ vs. respective control, n=3 for all groups.

4.3.3.2 Acetylcholine mediated protection against ipratropium induced myocardial injury is associated with attenuation of Erk1/2 phosphorylation

To determine whether ACh protects against ipratropium induced myocardial injury via a mechanism which involves phosphorylation of Erk1/2, western blot analysis was used to determine the expression levels in homogenised heart tissue samples, collected as previously described, in the presence of ipratropium (1×10^{-7} M) ± ACh (1×10^{-7} M). Figure 4.10 shows the results from this study.

A significant increase in levels of Erk1/2 phosphorylation was observed at all time points following ipratropium (1×10^{-7} M), ACh (1×10^{-7} M) and concomitant administration of ipratropium and ACh (concentrations as before) ($p<0.05$ or 0.01, values and statistical significance depicted in Figure 4.10 (**D**).

There was no significant change in Erk1/2 phosphorylation in comparison with the respective ipratropium (1×10^{-7} M) group following 15 or 120 minutes reperfusion. However, following 5 minutes reperfusion, both the ACh and ipratropium + ACh treatment groups showed a significant decrease in Erk1/2 (phosphorylation in comparison with the ipratropium alone group). However, despite reiterating, neither of these groups were statistically different from the untreated control.

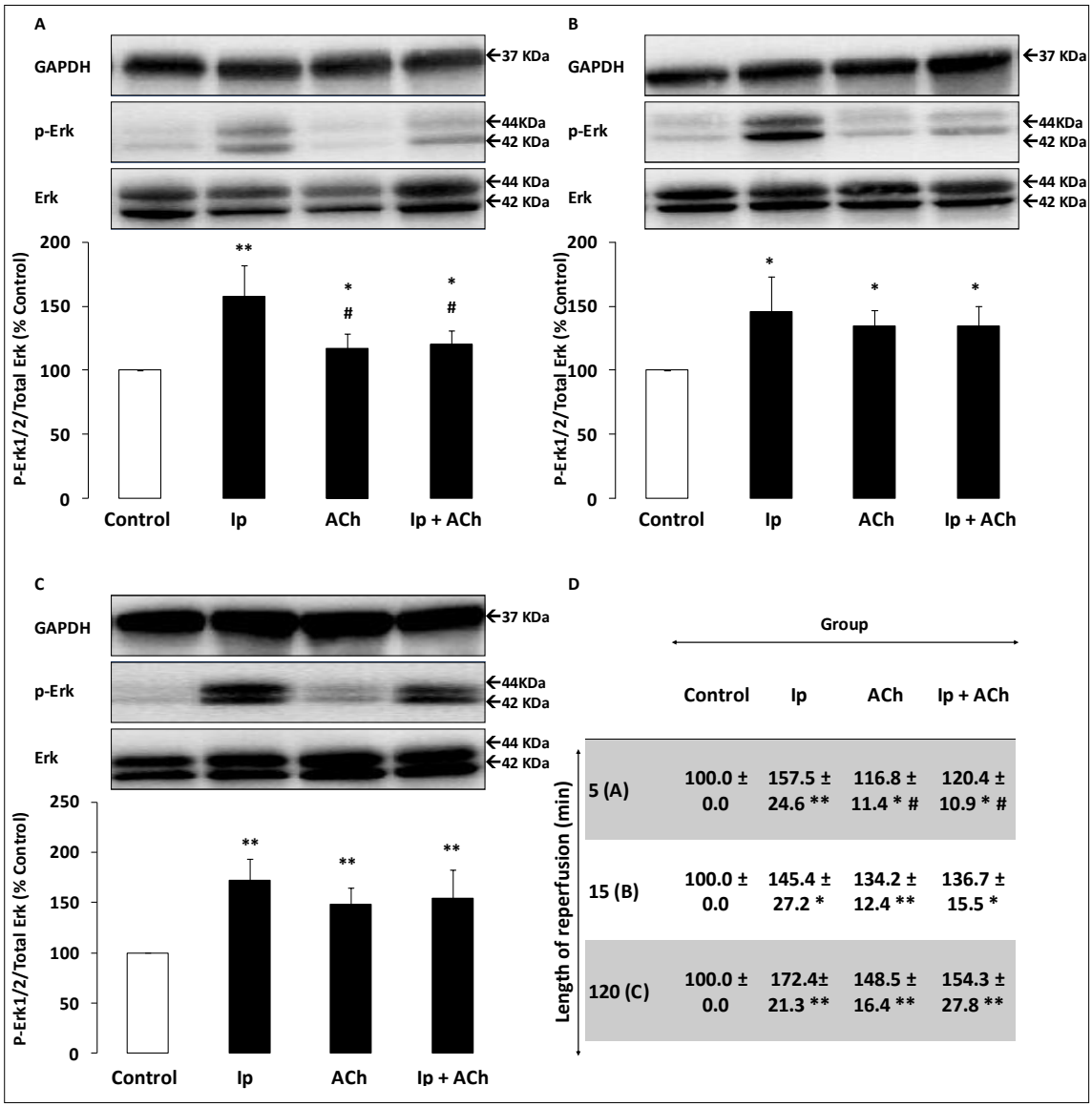


Figure 4.10 The effects of ipratropium (1 x 10^{-7} M) ± acetylcholine (1 x 10^{-7} M) treatment on the levels of phosphorylated Erk1/2 following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-Erk1/2 and total Erk1/2 for each time point shown above figures with treatment groups in the following order for all blots: Control, Ip, ACh and Ip + ACh. All values expressed as mean + SEM of p-Erk1/2/Erk1/2 as a percentage of the control (**D**). *p<0.05 and **p<0.01 vs. respective control, #p<0.05 and vs. respective Ip treated group, n=3 for all groups.

4.3.4 Effect of ipratropium treatment on the levels of JNK phosphorylation following ischaemia/reperfusion

JNK is a stress activated kinase with a well defined role in I/R injury. Following phosphorylation, JNK permits myocyte loss through activation of apoptosis and autophagy. JNK is also a downstream effector of muscarinic receptor signalling (Fujino et al. 2000, Hornigold et al. 2003) Of particular interest in the context of the myocardium, is the observation for potential cross-talk between the signalling pathways of the M_2 and M_3 subtypes via a mechanism which involves JNK (Hornigold et al. 2003).

4.3.4.1 Ipratropium induced myocardial injury is associated with a down-regulation of JNK phosphorylation

Tissue was harvested, as previously described, following 5, 15 and 120 minutes reperfusion. Figure 4.11 presents data from Western blot analysis where ipratropium ($1x10^{-9}$ M - $1x10^{-7}$ M) was administered throughout reperfusion. At all time points, there was a decrease in JNK phosphorylation following ipratropium administration (all concentrations). However, this was only significant following 15 minutes reperfusion in comparison with the respective untreated control (Figure 4.11 (**B**), values in 4.11 (**D**), $p<0.05$) and also for the group treated with 1×10^{-8} M ipratropium following 120 minutes reperfusion (4.11 (**C**), 78.3 ± 10.6%, Ip, vs. 100.0 ± 0.0%, control, $p<0.05$).

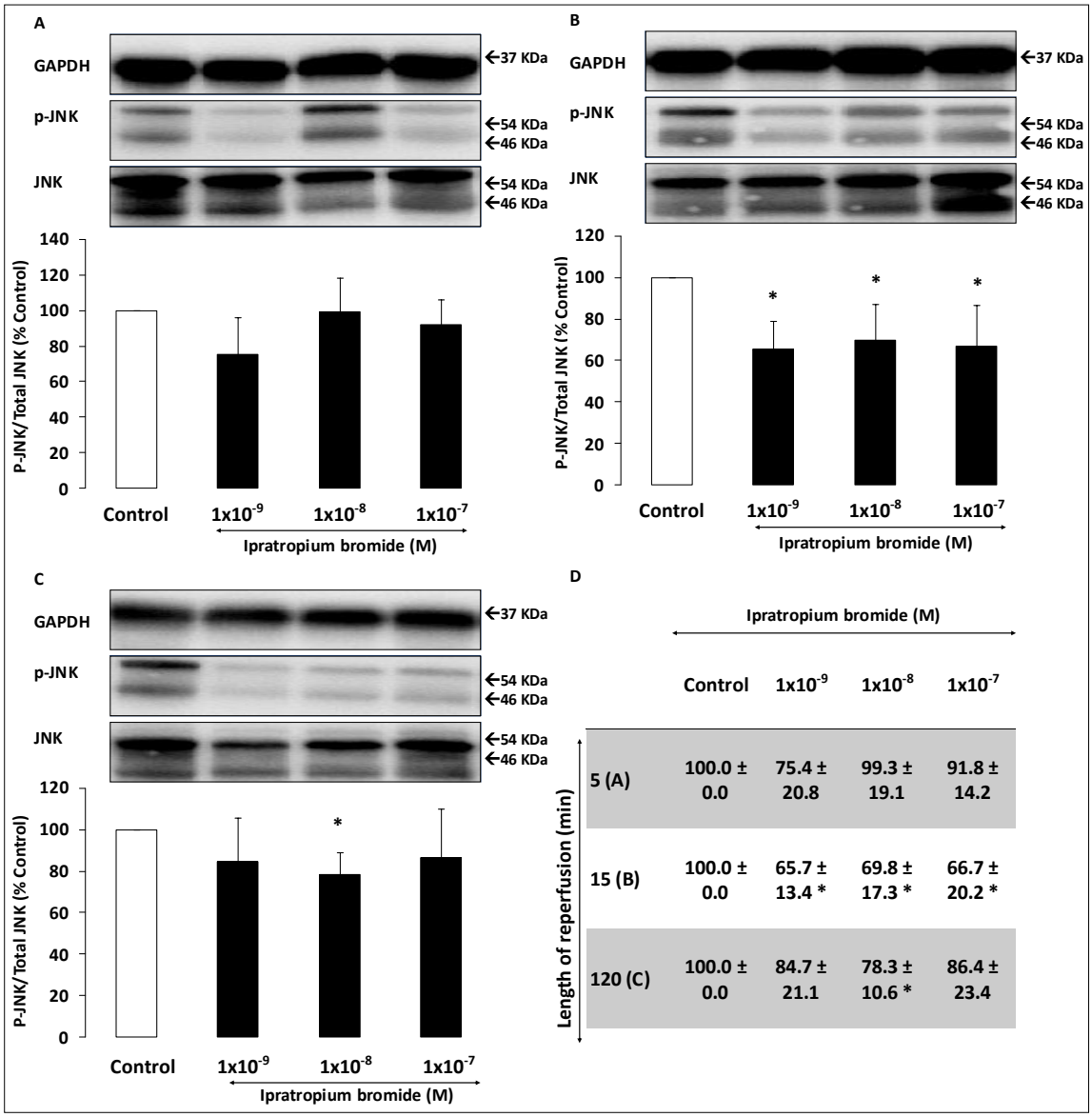


Figure 4.11 The effects of ipratropium (1 x 10^{-9} M – 1 x 10^{-7} M) treatment on the levels of phosphorylated JNK following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-JNK and total JNK for each time point shown above figures with treatment groups in the following order for all blots: Control, Ip (1x10^{-9} M), Ip (1 x 10^{-8} M), and Ip (1 x 10^{-7} M). All values expressed as mean + SEM of p-JNK/JNK as a percentage of the control (**D**). * $p<0.05$ vs. respective control, n=3 for all groups.

4.3.4.2 Acetylcholine mediated protection against ipratropium induced myocardial injury is associated with increased phosphorylation of JNK

Following administration of ipratropium ± ACh throughout 5 minutes of reperfusion, there was no significant difference in JNK phosphorylation between any of the treatment groups and the respective control (Figure 4.12 (**A**)).

At the 15 minute time point, there was a significant decrease in JNK phosphorylation in the group treated with ipratropium (66.7 ± 20.2%, ip, vs. 100.0 ± 0.0%, control, $p<0.05$). In contrast to this, there was a significant increase in JNK phosphorylation in the group treated with acetylcholine alone, in comparison with the untreated control (181.7 ± 19.4, ACh vs. 100.0 ± 0.0%, control, $p<0.05$). As well as this, in comparison with the group treated with ipratropium alone, there were statistical increases in JNK phosphorylation in the groups treated with ACh, both alone and in conjunction with ipratropium (Figure 4.12 (**B**), values in (**D**)). However, the group treated with ipratropium and ACh was statistically indistinct in comparison with the untreated control.

For the studies which looked at the effect of ipratropium and ACh following 120 minutes reperfusion, there was no significance between the control group and the groups treated with either ipratropium alone, or the group where ipratropium and ACh were co-administered. However, there was a statistical increase in JNK phosphorylation in the group treated with ACh alone in comparison with both the untreated control and the group treated with ipratropium alone (159.2 ± 17.5%, ACh, vs. 100.0 ± 0.0%, control and 86.4 ± 23.4%, Ip, $p<0.05$ and 0.01 respectively).

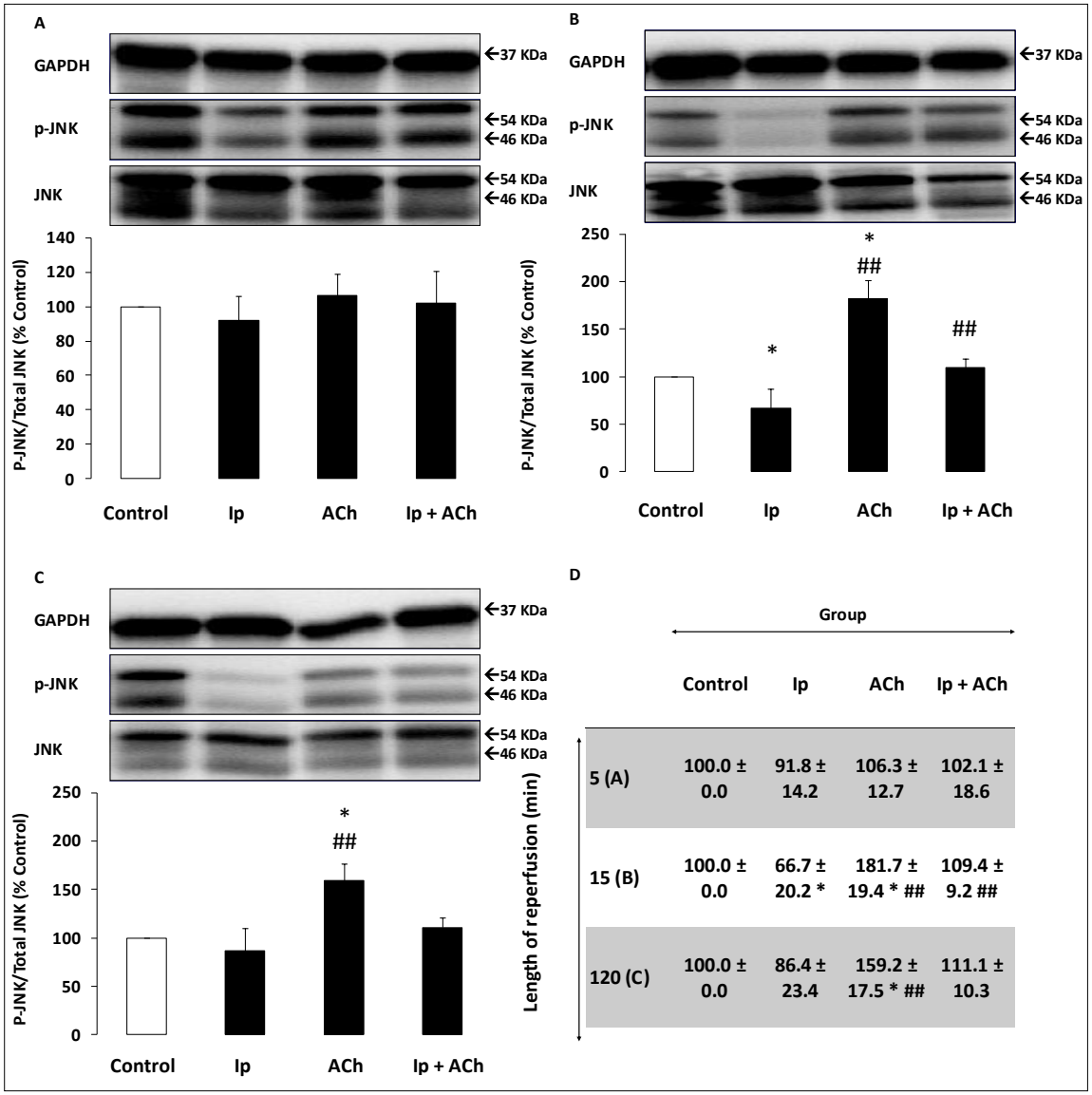


Figure 4.12: The effects of ipratropium (1 x 10^{-7} M) ± acetylcholine (1 x 10^{-7} M) treatment on the levels of phosphorylated JNK following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-JNK and total JNK for each time point shown above figures. Treatment groups in the following order for all blots: Control, Ip, ACh and Ip + ACh. All values expressed as mean + SEM of p-JNK/JNK as a percentage of the control (**D**). * $p<0.05$ vs. respective control, ## $p<0.01$ vs. respective Ip treated group, n=3 for all groups.

4.3.5 BAD

BAD, a BH-3 only domain member of the Bcl-2 family, is a potent mediator of apoptosis. Phosphorylation of BAD confers anti-apoptotic actions whereas, upon desphosphorylation, BAD is permitted to heterodimerise with Bcl-2 and Bcl-xL and initiate intrinsic apoptosis via Bax/Bak channel formation on the outer mitochondrial membrane fully described in section 1.4.2).

4.3.5.1 Ipratropium induced myocardial injury is associated with decreased phosphorylation of BAD

Flow cytometry was employed using isolated primary ventricular myocytes which had been exposed to the hypoxia/re-oxygenation protocol with administration of all drugs (ipratropium (1×10^{-9} M – 1×10^{-6} M), ACh (1×10^{-7} M), atropine (1×10^{-7} M) and ipratropium (1×10^{-7} M) administered concomitantly with either ACh or atropine (concentrations as before)) occurring throughout re-oxygenation.

The results from these flow cytometry studies are presented in Figure 4.13. Following ipratropium (1×10^{-9} M – 1×10^{-6} M) administration, there was a decrease in levels of BAD phosphorylation, which was significant in comparison with the control group, following 1×10^{-6} M and 1×10^{-7} M ipratropium (71.4 ± 9.5%, 1×10^{-6} M and 72.6 ± 11.7, 1×10^{-7} M, vs. 100.0 ± 0.0%, control, $p<0.05$, Figure 4.13 (**A**)). The decrease in BAD phosphorylation was also observed following 1×10^{-7} M atropine administration (80.1 ± 14.5% vs. 100.0 ± 0.0%, control, $p<0.05$, Figure 4.13 (**B**)).

4.3.5.2 Acetylcholine mediated protection against ipratropium induced myocardial injury is associated with increased phosphorylation of BAD

The administration of ACh significantly increased the levels of BAD phosphorylation in comparison with the control group (132.9 ± 10.6% vs. 100.0 ± 0.0%, control, $p<0.05$). Interestingly, co-administration of ACh with either ipratropium or atropine was capable of increasing the levels of phosphorylated BAD to levels which were statistically insignificant from the untreated control group (104.1 ± 9.8% (Ip + ACh) and 103.0 ± 12.5% (Atr + ACh). However, in both of these treatment groups, the change in level of BAD phosphorylation was also statistically indifferent from the groups treated with ipratropium or atropine alone, respectively (Figure 4.13 (**C**)).

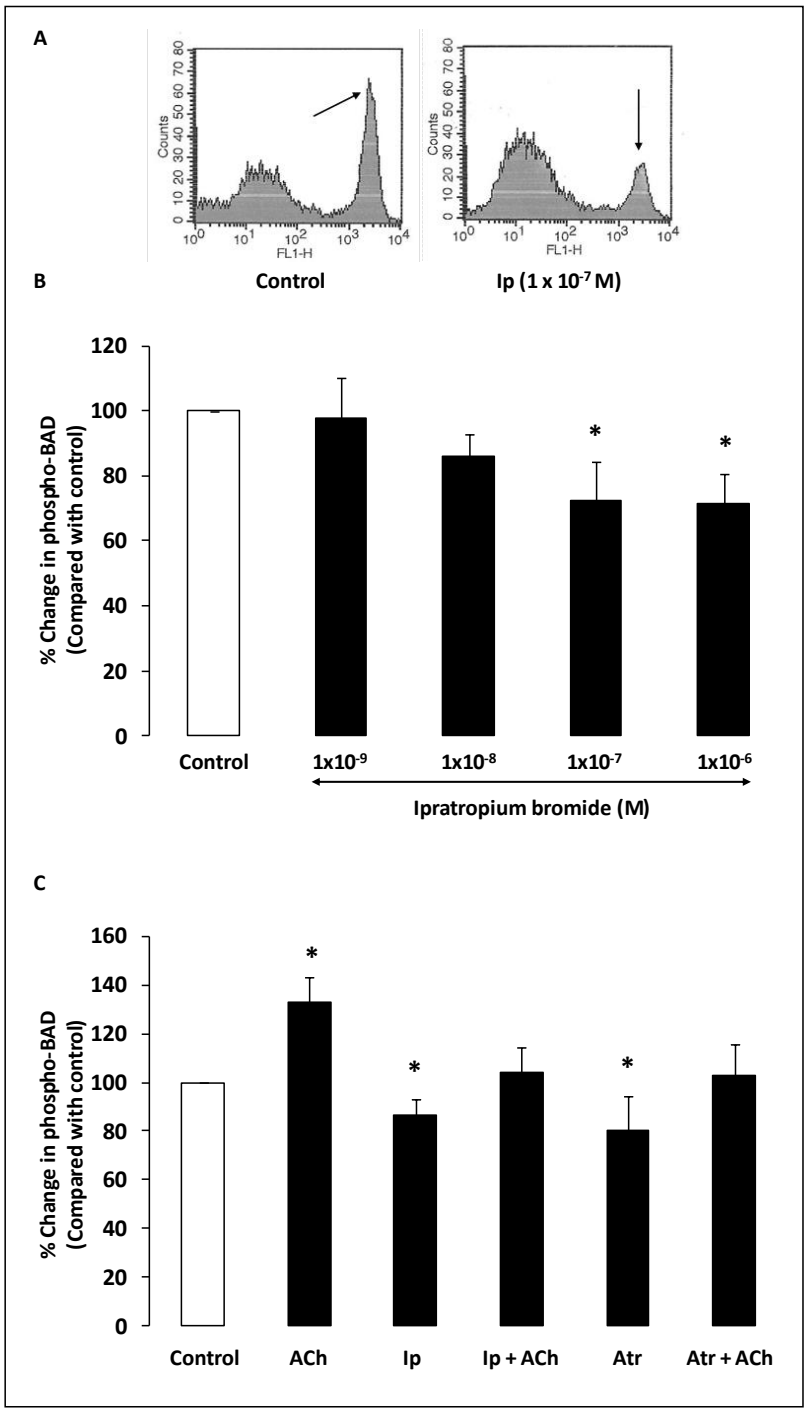


Figure 4.13: (A) Representative flow cytometric histograms to show the difference in BAD phosphorylation following ipratropium administration in comparison with the untreated control. **(B)** Effect of ipratropium (Ip, 1 x 10^{-9} M – 1 x 10^{-6} M) on levels of BAD phosphorylation in isolated rat ventricular myocytes following hypoxia/re-oxygenation, as determined by flow cytometric analysis. **(C)** Effect of ipratropium (Ip, 1 x 10^{-7} M) or atropine (Atr, 1 x 10^{-7} M) ± acetylcholine (ACh, 1 x 10^{-7} M) on levels of phosphorylated BAD. All drugs administered at the onset of, and throughout, re-oxygenation. Values are expressed as mean + SEM. * $p<0.05$ vs. respective untreated control group, n = 10 for all groups.

4.4 Chapter Discussion

The primary findings of this chapter are that ipratropium induced myocardial injury via a mechanism which involves up-regulation of the pro-survival kinases Akt and Erk1/2 as well as a decrease in phosphorylated JNK and BAD. This was also associated with a decreased time for depolarisation and hypercontracture, indicative of mPTP opening and myocyte death due to lack of ATP, respectively. In all models, the addition of ACh was shown to have, overall, opposing effects to ipratropium which was accompanied by a reduction in myocardial injury in comparison with control groups. Also, ACh was capable of eliciting protection against ipratropium injury when ipratropium and ACh were co-administered.

The opening of the mPTP during reperfusion has been shown to promote both necrotic and apoptotic cell death (Crompton, 1999). The conditions of oxidative stress, as associated with I/R, lead to mPTP opening (Halestrap, 2009, Halestrap and Pasdois, 2009, Hausenloy et al., 2002). The oxidative stress model used in this work measured depolarisation, indicative of mPTP opening and also subsequent hypercontracture, rigor of cardiac myocytes due to ATP depletion. The results from the oxidative stress myocyte model showed that administration of ipratropium rendered myocytes less resilient to injury following TMRM free radical generation. This was quantified by a reduction in time to both depolarisation and hypercontracture following ipratropium treatment. In contrast, acetylcholine exerts a protective effect via inhibition of the mPTP (Okorie et al. 2011, Sun et al. 2010). This has been attributed to a mechanism which links RISK signalling (including activation of Akt and Erk1/2) (Krieg at al. 2003). It has also been shown that acetylcholine elicits a cardioprotective response in I/R via a

mitochondrial ATP-sensitive potassium channel leading to blocking of mPTP opening (Sun et al. 2010). These previous experimental observations support the findings reported in this study.

In addition, the ability for acetylcholine to attenuate the ipratropium induced reduction in time to depolarisation and hypercontracture indicates involvement of the mPTP in ipratropium induced myocardial injury following oxidative stress. Despite all treatment groups (Ip, ACh and Ip + ACh) showing significance in comparison with the untreated control group at depolarisation, the differences between treatment groups and control were not as marked when hypercontracture was measured. Hypercontracture is rigour contracture of cardiac myocytes due to ATP depletion, however, depolarisation is indicative of loss of mitochondrial membrane integrity due to opening of the mPTP and subsequent outer membrane rupture (Halestrap 2010).

The opening of the mPTP has been described as a "point of no return" such that, once this event has occurred, cells are committed to death, either by necrosis or apoptosis (Baines 2009). Therefore although it is interesting that the times to hypercontracture vary less among groups, it is the time to depolarisation which has determined the fate of the myocyte and ostensibly this is more important as, following depolarisation the mitochondrion will no longer be capable of producing its own ATP, and also quickly deplete ATP levels from surrounding, still functioning mitochondria, initiating the first, irreversible steps towards a cellular death pathway. The phenomenon of mitochondrial depolarisation occurs as a consequence of mPTP opening. Within the context of a healthy mitochondrion, H^+ is produced following electron transport through the redox complexes. These protons are transported across the inner mitochondrial membrane

(IMM) and, thus, H^+ accumulates within the intermembrane space, thereby causing a membrane potential across the IMM. During regular oxidative phosphorylation, the protons flow back into the matrix and are used by ATPase in ATP synthesis. However, under the pathological conditions of ischaemia, deregulation of Ca^{2+} and the associated lack of oxygen causes disruption to the electron transport chain which subsequently results in mPTP opening at the onset of reperfusion (Andrews, Royse and Royse 2012). This leads to rapid depolarisation of the IMM, due to H+ flow into the matrix independent of ATP production, which is accompanied by uncoupling of oxidative phosphorylation and, therefore rapid ATP depletion. Following mPTP opening, solute and water influx into the mitochondrial matrix leads to rapid swelling and eventual rupture of the outer mitochondrial membrane. In combination, these constitute irreversible mitochondrial damage which determines the fate of the myocyte to death by either necrosis or apoptosis (Halestrap 2009).

In a sense, the time to hypercontracture is irrelevant as myocyte death following depolarisation is inevitable. Hypercontracture occurs as a result of excessive contractile activation following the onset of reperfusion due to the sudden restoration of oxidative phosphorylation and ATP production. However, intracellular Ca^{2+} levels are elevated during this time (Padilla et al. 2000). In this work it is postulated that the decrease in variation following hypercontracture is attributed to the time for Ca^{2+} levels to reduce after ischaemic injury, or oxidative stress, and this is dependent on the restoration of correct sodium-calcium exchanger (NCX) and inositol tri-phosphate receptor (IP3R) function. Despite the severity of the ischaemic injury, this time frame does not significantly change. In order to further elucidate the involvement of the

mPTP in ipratropium induced myocardial injury, the immunosuppressant and mPTP blocker, cyclosporin A was also investigated in the context of I/R following ipratropium treatment (fully investigated in Chapter 5).

The data presented here reveal that ipratropium elicits cardiotoxic effects and support the previously published ability of acetylcholine to protect against the detrimental effects of I/R. In addition to this, the ability for acetylcholine to partially abrogate the ipratropium induced injury, at these concentrations, implies a specific role for muscarinic signalling in ipratropium induced myocardial injury. In particular, acetylcholine has been shown to promote cellular viability, differention and survival following a cellular insult, such as I/R through activation of GPCR coupled signalling leading to activation of Akt (This includes activation of Akt following muscarinic stimulation by ACh). However, although in this study, there was an observed increase in Akt phosphorylation following simulated I/R (5, 15 and 120 mins reperfusion) and ACh administration, this was not statistically distinct from the respective, untreated control groups.

It is well-documented that Akt activation is cytoprotective following various cellular stresses, such as I/R injury (Li et al. 2011, Liu et al. 2011a, Wang et al. 2012), through its inclusion in RISK pathway signalling (Cohen et al. 2001, Qin, Downey and Cohen 2003, Zang, Sun and Yu 2007). However, in this work, despite having observed an increase in myocardial injury following I/R, at all the time points measured (5, 15 and 120 minutes of reperfusion), there was an increase in Akt phosphorylation following ipratropium (1×10^{-9} M – 1×10^{-7} M) administration. This observation corresponds to previously published data which associate Akt phosphorylation with a reduction in

myocardial injury following I/R (Miyamoto, Murphy and Brown 2009, Su et al. 2012, Zhang et al. 2011).

However, it has also been ascertained that constitutive over expression of Akt mediated cellular signalling is detrimental to cellular survival (Sussman et al. 2010). It is therefore postulated here that the mechanism of ipratropium induced myocardial injury that has been observed in this work is, in part, attributed to over stimulation of Akt signalling (Nagoshi et al. 2005, O'Neill and Abel 2005). However, following wortmannin treatment, in conjunction with ipratropium administration, although there was a significant decrease in Akt phosphorylation (as determined by Western blot anyalysis) this was not accompanied by a decrease in infarct size (as identified through Langendorff experiments).

Erk1/2, as with Akt, is also a downstream kinase of PI3K. In this study, the pattern of phosphorylation following I/R and ipratropium ± ACh, mirrored those observed in the Western blots which ascertained Akt levels. As with Akt, Erk1/2 has well documented cardio-protective properties via its involvement in the RISK pathway (Davidson et al. 2006, Rabkin and Tsang 2008). The proposed mechanism for action is that Erk1/2 activation is able to prevent dephosphorylation of BAD and, subsequently, restrict caspase activation due to inhibition of MOMP and apoptosome formation (Bullard et al. 2005). However, as with Akt, over expression of Erk1/2 has been shown to have detrimental effects. Therefore it is proposed here that ipratropium elicits myocardial damage following I/R via a mechanism which involved constituitive over-activation of Akt and Erk1/2, possibly due to over-activation of PI3K, the upstream regulator of both kinases.

With use of wortmannin, a PI3K inhibitor, via Western blot, it was elucidated that wortmannin can entirely abrogate the increases in Akt observed following ipratropium administration. However, in further Langendorff studies, this was not associated with a decrease in infarct size. This indicates that, although a mechanism involving up-regulation of Akt, and also Erk1/2 phosphorylation occurs following ipratropium treatment, this is not a specific determinant for the development of injury due to ipratropium and, therefore potentially gives rise to a role for necrosis, rather than apoptosis in ipratropium induced myocardial injury.

The activation of the pro-apoptotic MAPK, JNK, has been shown to elicit myocardial injury, through both apoptosis and autophage, following I/R injury (Xu et al. 2014). It has also been documented that muscarinic activation has been shown to inhibit JNK, therefore affording the activation of cyto-protective mechanisms (Wang et al. 2003). In this study, it was hypothesised that ipratropium administration may block this protective mechanism and lead to an up-regulation of JNK. However, from the results presented in this chapter, (with the exception of the 15 minutes time point) there was no significant change in JNK phosphorylation in comparison with the untreated control groups. Further to this, ACh administration was shown to constitutively increase levels of JNK phosphorylation. As JNK signalling couples to autophagy as well as apoptosis, it is possible that the observed increases in JNK following ACh couple to an autophagy phenotype, rather than apoptosis, and thereby may act as an endogenous protective mechanism. It has previously been demonstrated that, following oxidative stress, artificial elevation of JNK can promote autophagy rather than apoptosis in order to assist in salvage of functional cells (Wu, Wang and Bohmann 2009).

It was also shown that levels of BAD phosphorylation were significantly elevated following ACh administration, but significantly reduced following ipratropium, or atropine, treatment. Co administration of ACh with either ipratropium or atropine ameliorated this effect. The ability for both atropine and ipratropium, in conjunction with ACh, to elicit similar effects indicates that it is a specific action on muscarinic signalling pathways that is responsible for the observed ipratropium induced myocardial injury, as both a non-selective muscarinic antagonists. BAD is an integral member of the Bcl-2 family that, following de-phosphorylation, potently couples to an apoptotic mechanism. These results support this as, in the groups treated with ACh, an increase in BAD phosphorylation was observed which was associated with cardio-protection. It has also been previously shown that, via a PI3K-dependent mechanism, phosphorylation of Akt and BAD are able to promote cellular survival and proliferation (Kuwahara et al. 2000). The ability for both ipratropium and atropine to reduce levels of phosphorylated BAD indicates that there is a role for apoptosis in anti-muscarinic mediated myocardial injury following I/R.

Despite previous findings in Chapter 3 that, within the *in vitro* models employed in these studies, endogenous levels of acetylcholine were present, from the work in this chapter, it is hypothesised that ipratropium is capable of blocking the cytoprotective properties of ACh at the cardiac muscarinic receptors, therefore promoting cellular mechanisms which lead to cell death. The ability for exogenous ACh to abrogate some of this observed injury implies that it is a mechanism which involves disruption of muscarinic signalling.

Chapter 5 Cyclosporin A protects against ipratropium induced myocardial injury following ischaemia/reperfusion and oxidative stress

5.1 Chapter introduction and purpose

Although the mechanism by which ischaemia/reperfusion (I/R) injury occurs has not been fully elucidated, there is increasing evidence for the involvement of the mitochondrial Permeability Transition Pore (mPTP). First reported in 1987 (Crompton, Costi and Hayat 1987), the mPTP is a non-specific pore in the mitochondrial membrane which opens at the onset of reperfusion. The mPTP is a major determinant in myocyte fate following I/R (Shanmuganathan et al. 2005).

In 1988, it was first discovered that Cyclosporin A (CsA), previously used as an immunosuppressive agent, was capable of potently inhibiting the opening of the mPTP (Crompton, Ellinger and Costi 1988). It has since been confirmed that CsA binds with high affinity to Cyclophilin D, a key component of the mPTP, thus inhibiting binding of Cyclophilin D to adenine nucleotide translocase and preventing subsequent opening of the mPTP (Basso et al. 2005). The successful delay in mPTP opening exerted by CsA has since elicited a multitude of studies to identify its potential use as a cardioprotective agent (Cohen, Yang and Downey 2008, Crompton 1999, Halestrap 2009, Halestrap and Pasdois 2009, Hausenloy, Boston-Griffiths and Yellon 2011). Alongside prevention of mPTP opening, the activation of the reperfusion injury salvage kinase (RISK) pathway is also a main target for cardioprotection (Hausenloy and Yellon 2004), The pro-survival RISK pathway consists of the kinase signalling cascades, phosphatidylinositol-3-OH

kinase (PI(3)K)-Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2) (Davidson et al. 2006). It was previously unknown whether prevention of mPTP opening and the RISK pathway were connected, however it has been shown that, in the context of insulin mediated cardioprotection, RISK pathway activation is necessary for protection by delaying opening of the mPTP (Davidson et al. 2006).

More recently, acetylcholine mediated cardioprotection (in both pre- and post-conditioning) has been associated with activation of the RISK pathway (Li et al. 2011, Zhao et al. 2010). Also, acetylcholine induced cardioprotection has been shown to be abolished by atractyloside, a known opener of the mPTP. This indicates an integral role of the mPTP in acetylcholine mediated cardioprotection (Sun et al. 2010a). This was supported by the results presented in Chapter 4, where acetylcholine was capable of delaying the onset of mitochondrial depolarisation and hypercontracture when administered alone and significantly abrogating the observed reduction in time to both depolarisation and hypercontracture associated with ipratropium administration. In addition to this, significant changes in phosphorylated Akt and Erk1/2 in comparison with ipratropium administration were observed with acetylcholine treatment (both alone and in conjunction with ipratropium).

This study aimed to identify whether the previously observed exacerbation of myocardial injury attributed to ipratropium (1×10^{-7} M) administration was abrogated when co-administered with CsA (2×10^{-7} M). Ipratropium and CsA were administered concomitantly using the Langendorff model of I/R and also simulated I/R in primary rat cardiac myocyte models to identify myocyte viability (via MTT (3-(4,5-Dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide) assay) and the involvement of apoptosis, necrosis, cleaved caspase-3 and phospho-BAD (evaluated by flow cytometry).

To further elucidate the involvement of the mPTP we used a cardiac myocyte model of oxidative stress (as previously described in Chapter 4) where the effect of CsA, when administered with ipratropium, was explored. In Chapter 4, it was established that ipratropium induced myocardial injury was associated with significant increases in the phosphorylation of the RISK pathway proteins Akt and Erk1/2. Western blot analysis was subsequently carried out to identify whether CsA ± ipratropium administration had an effect on levels of phosphorylated Akt, Erk1/2 or JNK.

5.2 Methods

60 adult, male, Sprague-Dawley rats (300 g ± 50 g body weight) were used throught the experiments detailed in this chapter. All experiments were subject to the exclusion criteria detailed in Chapter 2, section 2.8. For all data, results are presented as group means + standard error of the mean (SEM). For the Western blotting, MTT assay and flow cytometry data, values have been normalised to the respective untreated control, which is represented as 100%. Statistical differences were ascertained with the use of one-way ANOVA, with the exception of haemodynamics where a two-way ANOVA was employed, and Fisher's LSD post-hoc test.

5.2.1 Isolated perfused rat heart model

Following sacrifice by cervical dislocation and excision, rat hearts were mounted on Langendorff perfusion apparatus. For all experiments, the Langendorff model of I/R was used. Ipratropium (1×10^{-7} M) ± CsA (2×10^{-7} M) were administered after 55 mins, the onset of reperfusion. At the end of the experimental protocol, infarct size to risk ratio (I/R %) was determined via Evans blue and TTC staining. Throughout all experiments, left ventricular developed pressure (LVDP, mmHg), heart rate (HR, beats.min^{-1}) and coronary flow (CF, ml.min^{-1}) were measured to assess the stability of the hearts (as described in 2.3.3).

5.2.2 Analysis of adult rat ventricular myocytes via MTT assay

Isolated adult rat ventricular myocytes underwent two hours hypoxia after which, at the onset of re-oxygenation, ipratropium (1 x 10^{-7} M), CsA (2 x 10^{-7} M) and Ip + CSA (concentrations as previously stated) were administered for four hours, in the precence of MTT. Myocytes were subsequently lysed and analysed for MTT reductase activity.

5.2.3 Analysis of adult rat ventricular myocytes via flow cytometry

Isolated cardiac myocytes were subjected to the H/R protocol. Ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) were administered for 18 hours, following four hours hypoxia. At the end of the experimental protocol, apoptosis, necrosis and viable myocytes and levels of caspase-3 and BAD were assessed by flow cytometry.

5.2.4 mPTP model of oxidative stress

Following adhesion to laminin coated cover slips, adhered myocytes were incubated in microscopy buffer containing 3 x 10^{-6} M, tetramethylrhodamine methyl ester (TMRM) for 15 minutes. Cells were washed and randomly assigned to one of the following groups: untreated control, FCCP (2 x 10^{-6} M), Ip (1 x 10^{-7} M), CsA (2 x 10^{-7} M), or Ip + CsA (concentrations as before). Myocytes were viewed and analysed via confocal microscopy. Depolarisation (Dep) was measured as the time at which the TMRM

started to become evenly distributed throughout the cell and is indicative of the initiation of the mPTP opening. Subsequent hypercontracture (Hyp) of myocytes occurs shortly afterwards due to ATP depletion. The time to both Dep and Hyp were recorded.

5.2.5 Western Blotting

Tissue was harvested, following 5, 15 and 120 min reperfusion, as previously described. All drugs were administered at the onset of reperfusion. Following homogenisation and storage in suspension buffer, 60 µg of protein from was diluted with sample buffer and loaded into precast gels from samples of the following groups: untreated I/R control, ipratropium (1×10^{-7} M), CsA (2×10^{-7} M) and Ip + CSA (concentrations as before). After separation by electrophoresis, proteins were transferred onto PVDF membranes and probed for the phosphorylated forms of AKT, Erk1/2 and SAPK/JNK. To assess relative changes in protein levels, membranes were subsequently probed for the total, unphosphorylated forms of AKT, Erk1/2 and JNK and relative changes in phosphorylated protein were calculated as a percentage of total protein level. To ensure protein loading was equal, all membranes were also probed for GAPDH.

5.3 Results

5.3.1 The observed exacerbation of myocardial ischaemia/reperfusion injury is abrogated by cyclosporine A in the isolated perfused rat heart

To further elucidate the mechanism by which ipratropium exerted its cardiotoxic effect, cyclosporin A (CsA), a known inhibitor of the mPTP was used in conjunction with ipratropium. The present study was aimed at assessing whether CsA (2 x 10^{-7} M), a known cardioprotective agent, was capable of attenuating the observed ipratropium induced myocardial injury. In all experiments, a sub-maximal concentration of ipratropium (1 x 10^{-7} M) was used as has been previously demonstrated to elicit myocardial injury following I/R and H/R.

5.3.1.1 Effect of ipratropium ± cyclosporin A treatment on infarct development following ischaemia/reperfusion

The results presented in Figure 5.1 show the effect of cyclosporin A (2 x 10^{-7} M) administration at reperfusion. CsA significantly reduced infarct size in comparison with the control (22.9 ± 3.9 % (CsA, 2 x 10^{-7} M) vs. 51.8 ± 3.0 % (control), p,0.05). The observed increase in infarct size due to ipratropium treatment was significantly attenuated when administered in conjunction with cyclosporin A (75.1 ± 4.3 % (Ip, 1 x 10^{-7} M) vs. 62.7 ± 4.8 % (Ip + CsA, 1 x 10^{-7} M and 2 x 10^{-7} M, respectively), $p<0.05$). Values for all data presented are shown in table 5.1.

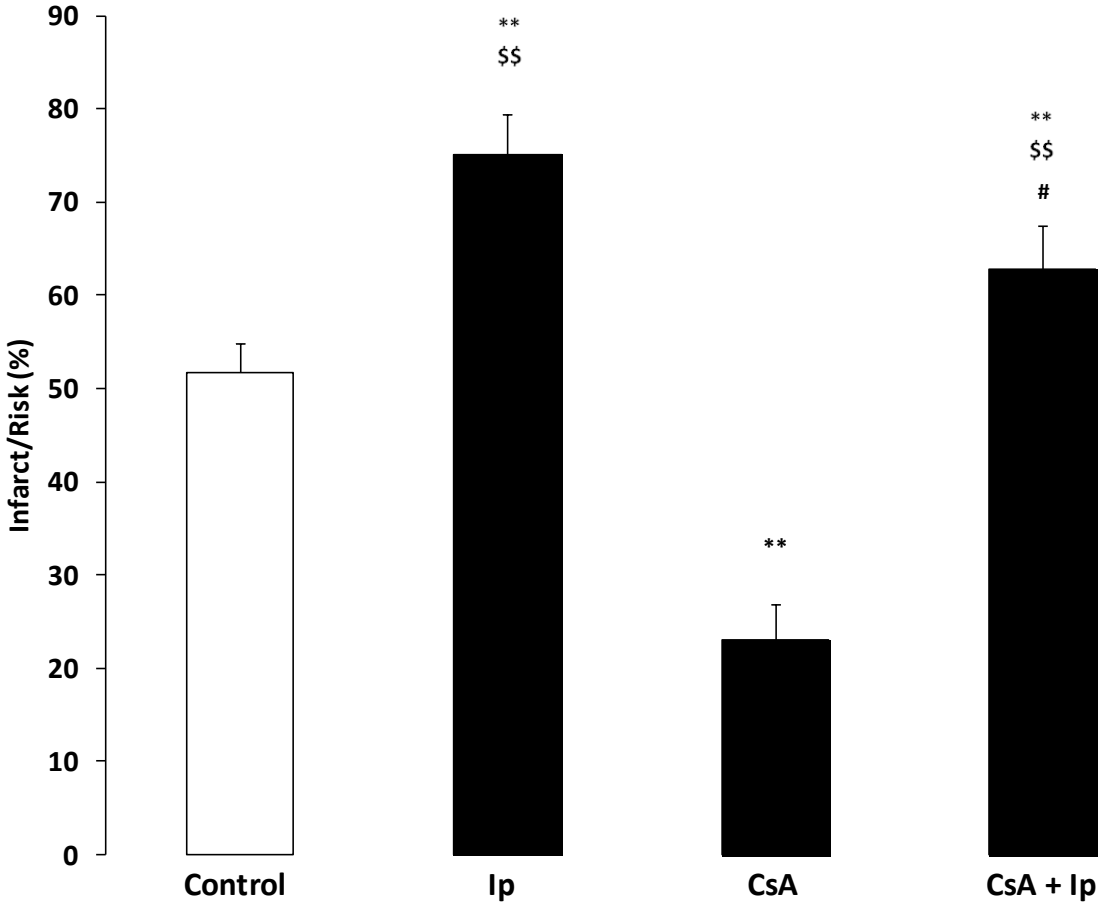


Figure 5.1: Infarct development in the risk zone following CsA ($2x10^{-7}$ M) treatment ± ipratropium ($1x10^{-7}$ M) in isolated perfused rat heart. CsA and ipratropium both administered at the onset of reperfusion. Results are presented as infarct/risk (%). **p<0.01 vs. control, $$p<0.01 vs. CsA, #p<0.05 vs. Ip. Results expressed as mean + SEM, n=6.

	Control	Ipratropium	CsA	Ip + CsA
		$1x10^{-7}$ M	$2x10^{-7}$ M	$1x10^{-7}$ + $2x10^{-7}$ M
I/R%	**51.8**	**75.1 ** $$**	**22.9 ****	**62.7 **$$ #**
SEM	**± 3.0**	**± 4.3**	**± 3.9**	**± 4.8**

Table 5.1: Infarct to risk percentage values in the isolated perfused rat heart following CsA ($2 x 10^{-7}$ M) treatment ± ipratropium ($1 x 10^{-7}$ M). CsA and ipratropium both administered at the onset of reperfusion. **p<0.01 vs. control, $$p<0.01 vs. CsA, #p<0.05 vs. Ip. Results expressed as mean ± SEM, n=6.

5.3.1.2 Haemodynamic parameters

All haemodynamic parameters were measured and expressed as previously described, and are presented in Figures 5.2 (LVDP), 5.3 (HR) and 5.4 (CF). There were no observed differences in LVDP, HR or CF in comparison with hearts in the control group with any treatment group (ipratropium (Ip, 1 x 10^{-7} M), CsA (2 x 10^{-7} M) or Ip + CsA (concentrations as before)) at any time point throughout the experimental procedure.

5.3.1.3 Left ventricular developed pressure (LVDP)

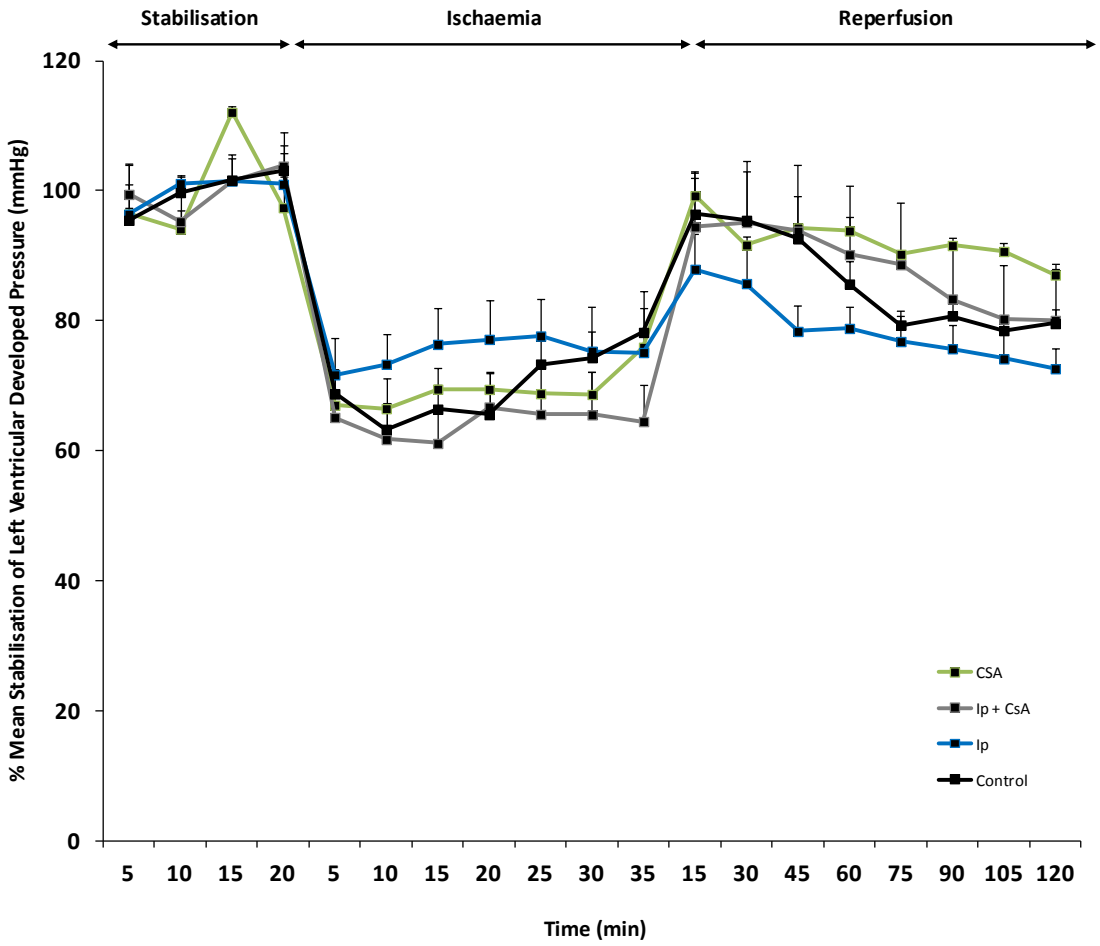


Figure 5.2 Changes in left ventricular developed pressure (mmHg) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. CsA ($2x10^{-7}$ M) ± ipratropium ($1x10^{-7}$ M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups.

5.3.1.4 Heart rate (HR)

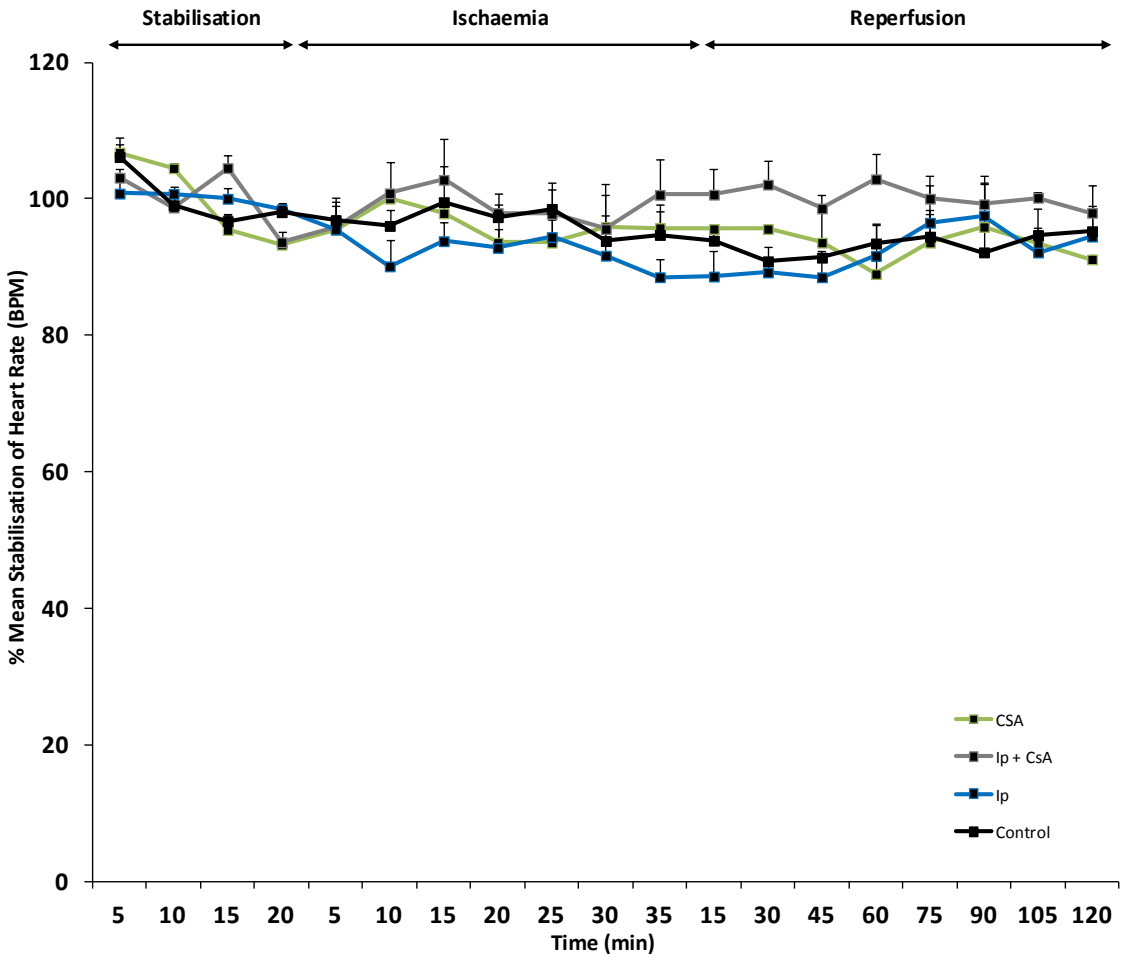


Figure 5.3 Changes in heart rate (bpm) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. CsA (2 x 10^{-7} M) ± ipratropium (1 x 10^{-7} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups.

5.3.1.5 Coronary flow (CF)

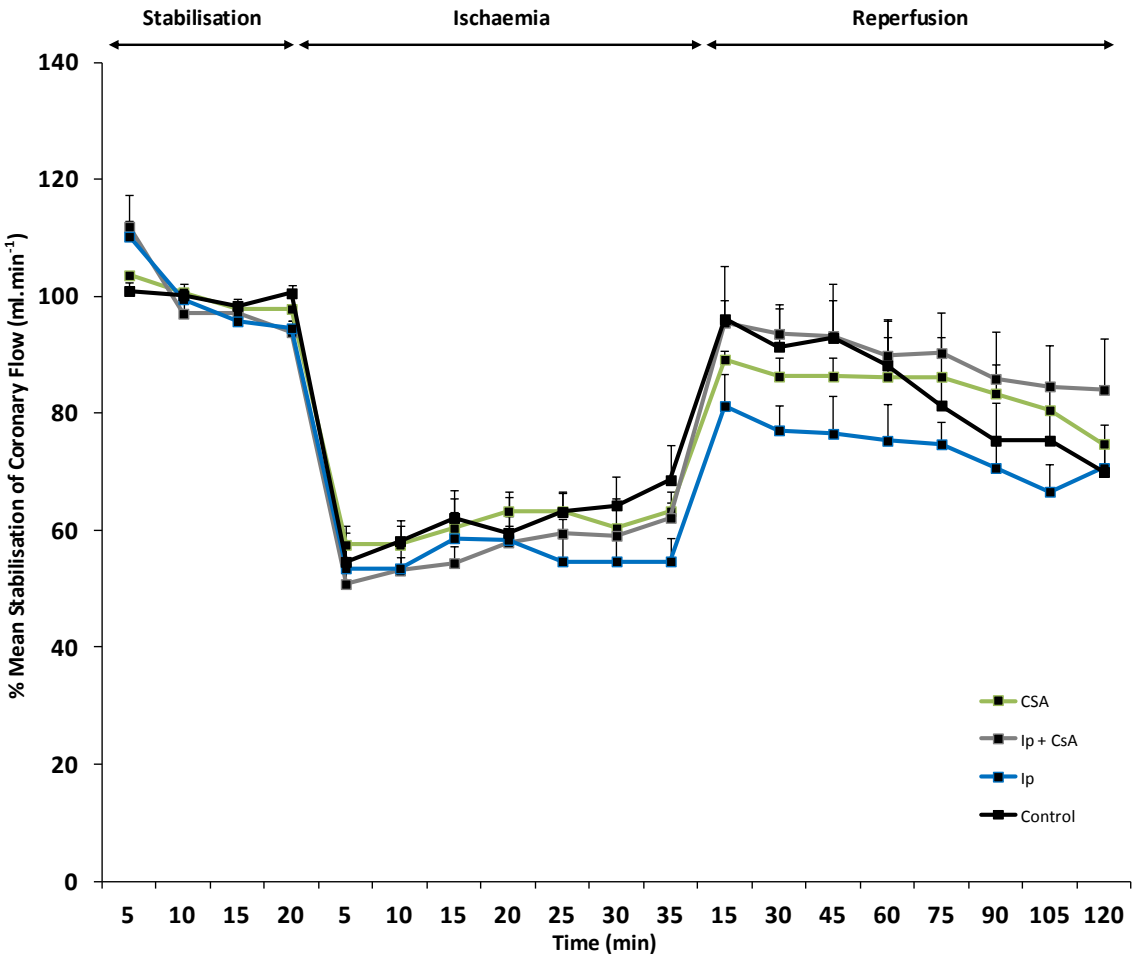


Figure 5.4 Changes in coronary flow (ml.min^{-1}) in isolated perfused rat hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. CsA (2 x 10^{-7} M) ± ipratropium (1 x 10^{-7} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 6 for all groups.

5.3.2 Assessment of MTT Reductase Activity following cyclosporin A ± ipratropium Treatment

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan by mitochondrial dehydrogenase, and therefore provides an assay for cellular viability. The cell viability assay was conducted in the presence and absence of CsA ± ipratropium (2 x 10^{-7} M and 1 x 10^{-7} M, respectively), as well as ipratropium alone (concentration as before), with all drugs being administered at the onset of re-oxygenation. Figure 5.5 shows percentage change in MTT reductase activity of the control in comparison with myocytes administered ipratropium ± CsA (concentrations and experimental groups as before).

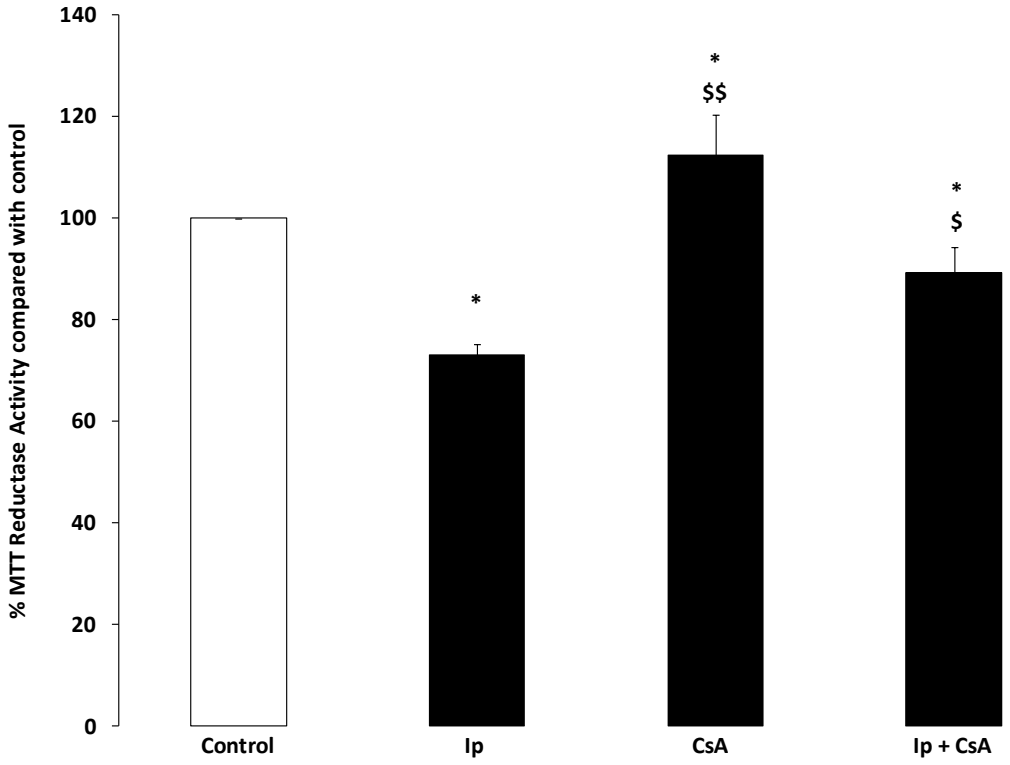


Figure 5.5: Assessment of myocyte viability via changes in MTT reductase activity in isolated rat ventricular myocytes following hypoxia/re-oxygenation. Administration of ipratropium (Ip, 1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) occurred at the onset of, and throughout, re-oxygenation. All groups were subjected to hypoxia/re-oxygenation protocol. Values are mean + SEM, n=6. *p<0.05 vs. control, $p<0.05 vs. Ip, $$p<0.01 vs. Ip n=6.

Administration of ipratropium at the onset of re-oxygenation produced a significant reduction in cell viability (73.0 ± 2.1% (Ip, 1 x 10^{-7} M) vs. 100 ± 0.0%, control, $p<0.05$). Treatment with CsA statistically increased cell viability in comparison with both the control and ipratropium treated groups (112.4 ± 7.9 (CsA, 2 x 10^{-7} M) vs. 100 ± 0.0%, control, $p<0.05$ and vs. 73.0 ± 2.1%, Ip, $p<0.01$). In the group where ipratropium was administered concomitantly with CsA, there was a significant increase in myocyte viability in comparison with the group treated with ipratropium alone (89.3 ± 4.9% (Ip + CsA) vs. 73.0 ± 2.1% (Ip), $p<0.05$). This is indicative that CsA is capable of partially compensating for the observed ipratropium induced loss of myocyte viability. However, although myocyte viability was significantly increased in the group where ipratropium and cyclosporin A were co-administered, the level of myocyte viability was still statistically less than in the untreated control group (89.3 ± 4.9% (Ip + CsA) vs. 100 ± 0.0%, control, $p<0.05$).

5.3.3 Assessment of apoptosis and necrosis in primary cardiac myocytes following hypoxia/re-oxygenation and ipratropium ± cyclosporin A treatment

In order to elucidate whether the observed protection of CsA against ipratropium mediated myocardial injury was via protection against necrosis or apoptosis, additional experiments were conducted which recorded, via flow cytometry, viable, apoptotic and necrotic myocyte populations, following ipratropium (Ip, 1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) treatment, at the onset of re-oxygenation. Apoptosis and necrosis were estimated by Annexin V and SYTOX® Green positive myocytes, respectively.

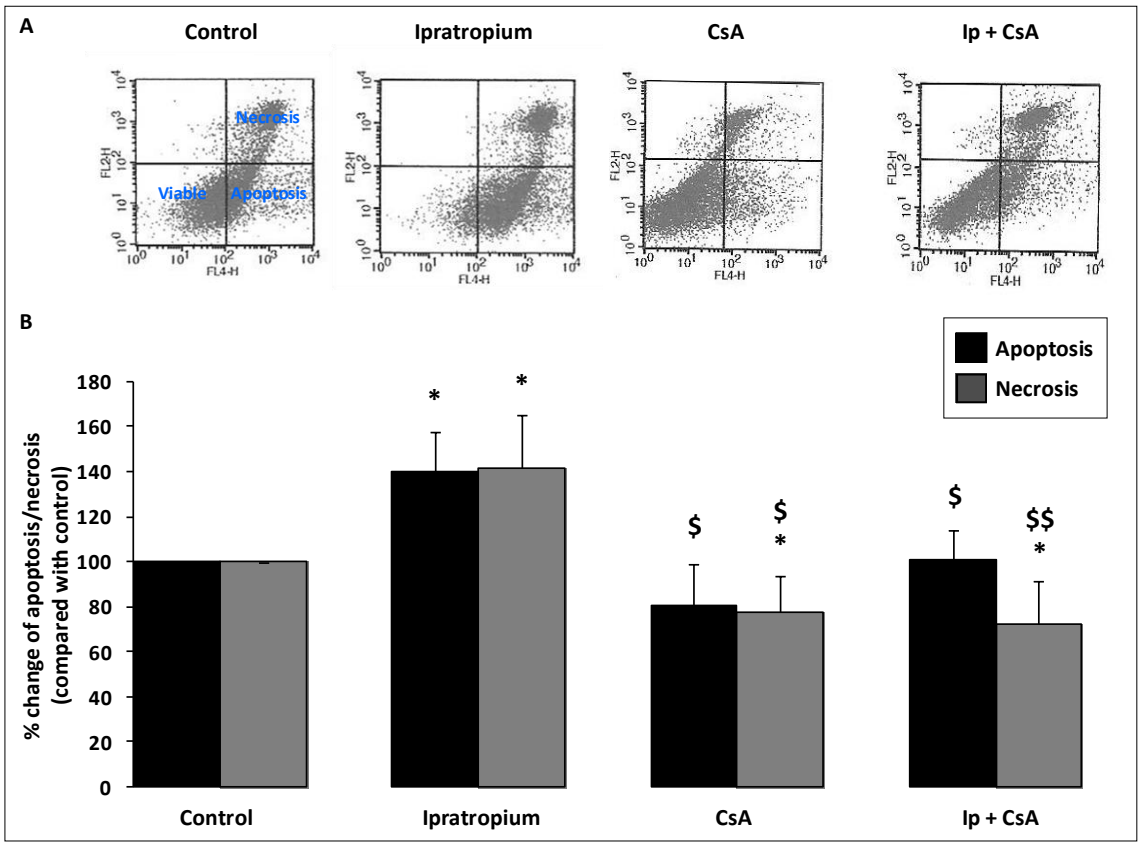


Figure 5.6: Assessment of apoptosis and necrosis in adult rat ventricular cardiac myocytes following H/R protocol and ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) treatment, administered at re-oxygenation. **A**, shows original representative flow cytometric scatter graphs presenting myocytes subjected to hypoxia and re-oxygenation in the absence (control) and presence of ipratropium (1 x 10^{-7} M), CsA (2 x 10^{-7} M) and ipratropium + CsA (Concentrations as before). **B**. Depicts normalised data for apoptotic and necrotic myocyte populations expressed as a percentage of untreated control. *p<0.05 in comparison with respective untreated control, $ p<0.05, $$ p<0.01 in comparison with respective ipratropium treated group. Values expressed as mean + SEM (n=8).

As shown in Figure 5.6, ipratropium (1 x 10^{-7} M) was capable of eliciting significant increases in both apoptosis (Annexin V positive) and necrosis (SYTOX® Green positive) in comparison with the respective untreated control (139.9 ± 18.1 %, Ip, vs. 100 ± 0.0 %, apoptosis and 141.6 ± 23.9 %, Ip, vs. 100 ± 0.0 %, necrosis, both p<0.05). As expected, a decrease in both apoptosis and necrosis was observed in the CsA (2 x 10^{-7} M) treated groups. However, only the decrease in necrosis produced a significant result in comparison with the untreated control (78.8 ± 15.4 % vs. 100 ± 0.0 %, p<0.05.

Interestingly, when ipratropium and CsA were co-administered, there was a statistical decrease in apoptosis in comparison with the group treated with ipratropium alone (139.9 ± 18.1 %, Ip, vs. 103.0 ± 10.6 %, Ip + CsA, $p<0.05$). There was also a significant decrease in the levels of necrosis in comparison with the group treated with ipratropium alone (141.6 ± 23.9 %, Ip, vs. 73.1 ± 18.5 %, Ip + CsA, $p<0.01$).

In groups treated with CsA (with and without ipratropium) the levels of apoptosis were not statistically distinct from the levels of apoptosis in the control group (80.8 ± 18.3 % (CsA) and 103 ± 10.6 % (Ip + CsA)). However, in contrast to this, in the CsA and Ip + CsA groups, a significant decrease in necrosis was observed in comparison with the control (78.8 ± 15.4 % (CsA) and 73.1 ± 18.5 % (Ip + CsA) vs. 100 ± 0.0 %, control, $p<0.05$). This is indicative that the action of CsA promotes protection via a mechanism which primarily prevents necrosis with only a small contribution to the prevention of apoptosis. It is well documented that cyclosporin A is capable of inhibiting mPTP opening during reperfusion, thereby promoting myocyte survival (Halestrap 2009). Upon mPTP opening, myocyte loss occurs via necrosis and also apoptosis (Yellon and Hausenloy 2007). These data suggest that ipratropium induced myocardial injury occurs via both necrosis and apoptosis and that cyclosporin A is capable of partially compensating for the necrotic component of this myocyte death.

5.3.4 Assessment of Caspase-3 levels in Primary Cardiac Myocytes following Hypoxia/Re-oxygenation and cyclosporin A ± Ipratropium Treatment

To further identify the involvement of apoptosis in CsA mediated protection against ipratropium induced injury, levels of cleaved caspase-3 were evaluated via flow cytometry, as described previously, in the presence of ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M). These data are presented in Figure 5.7.

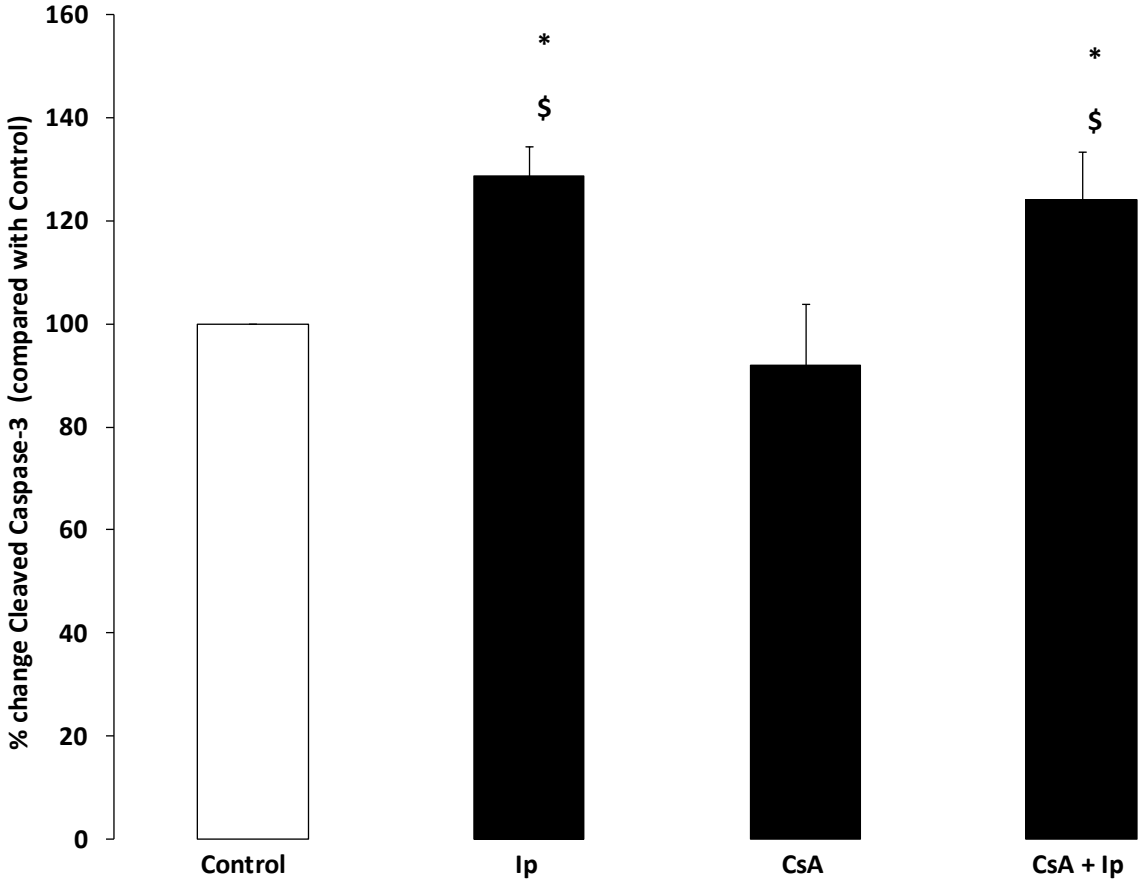


Figure 5.7: Effect of ipratropium (Ip, 1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) on cleaved caspase-3 levels in ventricular cardiac myocytes following hypoxia and re-oxygenation protocol. Ipratropium and CsA administered at the onset of, and throughout, re-oxygenation. *p<0.05 vs. untreated control, $p<0.05 vs. CsA (2 x 10^{-7} M). Results expressed as arithmetic mean, normalised to untreated control, + SEM, n=6.

Exposure of ventricular myocytes to ipratropium (1×10^{-7} M) throughout re-oxygenation was associated with a significant increase in levels of caspase-3 in comparison with the untreated control (128.7 ± 5.8 % vs. 100 ± 0.0 %, $p<0.05$), as previously shown in chapter 3. Addition of CsA (2×10^{-7} M) did not cause a significant difference in levels of caspase-3 in comparison with the control (91.9 ± 12.0 % vs. 100 ± 0.0 % (control)). The co-administration of ipratropium with CsA produced effects which were statistically indistinct from the group treated with ipratropium alone (128.7 ± 5.8 % (Ip) vs. 124.1 ± 9.4 % (Ip + CsA)) however were significantly higher than both the untreated control and group treated with CsA alone (124.1 ± 9.4 % (Ip + CsA) vs. 100 ± 0.0 % (control) and vs. 91.9 ± 12.0 % (CsA), both $p<0.05$). This is indicative that CsA does not protect against ipratropium induced myocardial injury via a mechanism which is dependent on a caspase-dependent apoptotic cascade. However, due to the observed decrease in apoptotic death following CsA administration (Fig. 5.6) it may be the case that the mode of CsA protection is primarily reliant on the mitochondria, or extracellular “death signals” which instigate apoptosis through a route which is not dependent on caspase-3.

5.3.5 Assessment of phospho-BAD levels in Primary Cardiac Myocytes following Hypoxia/Re-oxygenation and cyclosporin A ± Ipratropium Treatment

To further identify the involvement of apoptosis in CsA mediated protection against ipratropium induced injury, levels of phospho-BAD were evaluated via flow cytometry, as described previously, in the presence of ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M). These data are presented in Figure 5.8.

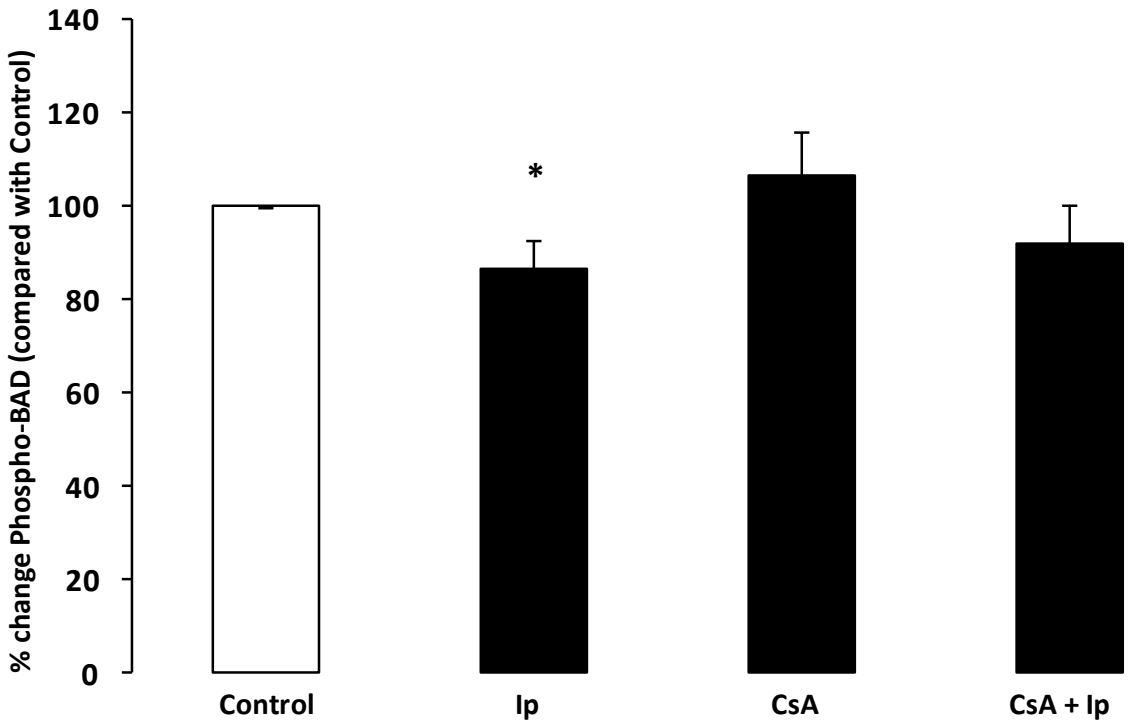


Figure 5.8: Effect of ipratropium (Ip, 1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) on levels of phospho-BAD in ventricular cardiac myocytes following hypoxia and re-oxygenation protocol. Ipratropium and CsA administered at the onset of, and throughout, re-oxygenation, *p<0.05 vs. untreated control. Results expressed as arithmetic mean + SEM, n=10.

BAD is a BH-3 only domain member of the Bcl-2 protein family which, in the context of a healthy cell, is phosphorylated by Akt. When phosphorylated, BAD dissociates with Bcl-2 and Bcl-xL (anti-apoptotic members of the Bcl-2 family) this permits the association of Bcl-2 with Bax, thereby inhibiting apoptosis. Dephosphorylation of BAD (frequently triggered by increases in Ca^{2+} and conditions of ischaemia/reperfusion) induces caspase-dependent and –independent apoptosis via a mitochondrial associated pathway due to heterodimeric formation with Bcl-2 and Bcl-xL.

When ipratropium was administered at the onset of re-oxygenation, there was a significant decrease in levels of phospho-BAD in comparison with the untreated control (86.3 ± 6.7 %, Ip (1×10^{-7} M) vs. 100.0 ± 0.0 %, control, $p<0.05$). CsA (2×10^{-7} M) administration (both alone and in conjunction with ipratorpium) produced effects which were statistically indistinct from the control groups (106.7 ± 9.5 % (CsA) and 92.1 ± 8.4 % (CsA + Ip)). This is indicative that ipratropium mediated myocardial injury is associated with dephosphorylation of BAD, thereby initiating apoptosis. CsA did not exhibit the ability to significantly protect against BAD de-phosphorylation. Also, there was no significant increase in phospho-BAD in comparison with the group treated with ipratropium alone and the group where CsA and ipratropium were co-administered.

5.3.6 The role of cyclosporin A and mPTP opening following ipratropium administration in a model of oxidative stress

As previously shown in Chapter 4, administration of ipratropium (1 x 10^{-7} M) was shown to reduce the time for depolarisation (indicative of mPTP opening) and subsequent hypercontracture (myocyte rigor due to ATP depletion), in a dose responsive manner, this was partially reversed by the co-administration of acetylcholine. To determine whether the mPTP also plays a role in the observed protection afforded by CsA, these experiments were repeated using ipratropium (1 x 10^{-7} M) in the presence and absence of CsA (2 x 10^{-7} M), a known inhibitor of the mPTP and cardioprotective agent.

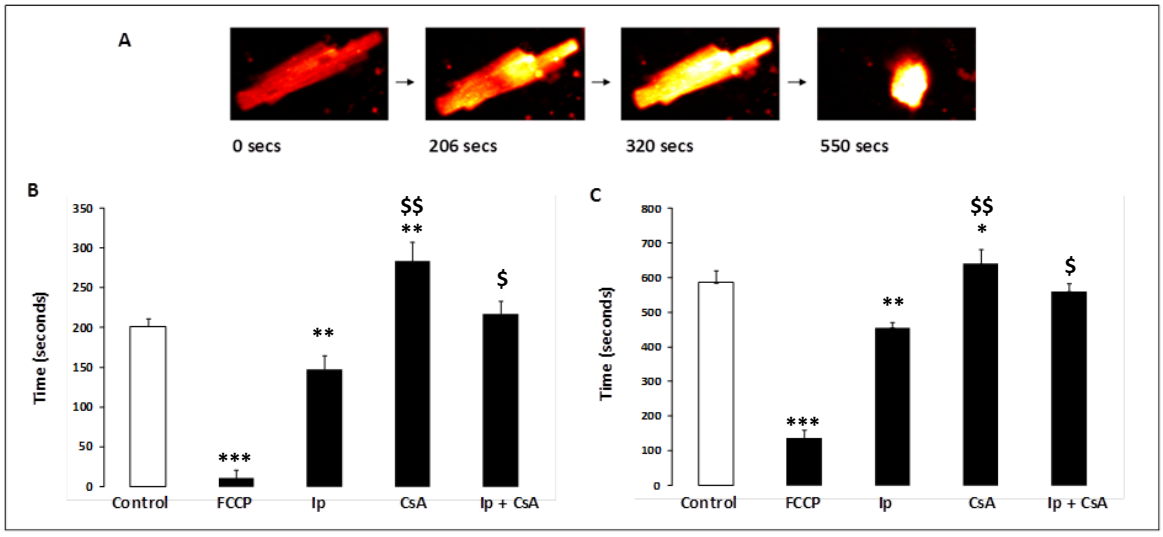


Figure 5.9: Effect of FCCP (2 x 10^{-6} M) and ipratropium (Ip, 1 x 10^{-7} M) ± CsA, (2 x 10^{-7} M) on time to depolarisation (**B**) and hypercontracture (**C**) under the conditions of sustained oxidative stress. *p<0.05, ** p<0.01, *** p<0.001 vs. respective control (white bars), $p<0.05, $$p<0.001 vs. respective Ip treated group. Values expressed as mean + SEM, n=6 animals, with between 10 and 15 myocytes measured, per animal, for each group.

	Control		FCCP (2×10^{-6} M)		CsA (2×10^{-7} M)		Ip (1×10^{-7} M)		Ip + CsA	
	Dep	Hyp	Dep	Hyp	Dep	Hyp	Dep	Hyp	Dep	Hyp
Time (s)	**201.2**	**585.3**	**11.3**	**136.9**	**283.1**	**638.9**	**147.5**	**455.2**	**217.1**	**559.0**
SEM	**9.9**	**33.9**	**9.8**	**22.9**	**24.0**	**41.0**	**17.5**	**15.3**	**16.4**	**23.8**

Table 5.2: Time taken, in seconds, for depolarisation and hypercontracture under the conditions of sustained oxidative stress following FCCP (2×10^{-6} M) and ipratropium (Ip, 1×10^{-7} M) ± CsA (2×10^{-7} M) treatment.

The model of mPTP opening was used to determine myocyte depolarisation and hypercontracture as established using TMRM loaded cells subjected to laser stimulated oxidative stress generation. As shown in Figure 5.9, time points measured are schematically represented (**A**) the time to depolarisation, 2nd panel, and hypercontracture, 4th panel, as visualised by confocal microscopy. Time for ventricular myocyte depolarisation (**B**) and subsequent hypercontracture (**C**) were recorded. FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) is known to disrupt oxidative phosphorylation and was included in the experiments as a positive control as it has previously been shown as a potent uncoupler of mitochondrial membrane potential. FCCP caused a significant reduction in time to both depolarisation and hypercontracture in comparison with the untreated control (11.3 ± 9.8 s vs. 201.2 ± 9.9 s and 136.9 ± 17.5 s vs. 585.3 ± 33.9 s, respectively, both $p<0.001$). As before, ipratropium (1×10^{-7} M) also significantly reduced time to both depolarisation and hypercontracture in comparison with the respective untreated controls, both $p<0.01$. When administered alone, CsA (2×10^{-7} M) was capable of significantly prolonging time to hypercontracture and depolarisation (283.1 ± 24.0 s vs. 201.2 ± 9.9 s and 638.9 ±

41.1 s vs. 585.3 ± 33.9 s, $p<0.01$ and $p<0.05$ respectively). Co-administration of ipratropium with CsA was capable of significantly attenuating the observed reduction in time to depolarisation (217.1 ± 16.4 s, Ip + CsA, vs. 147.5 ± 17.5 s, Ip, $p<0.05$) and hypercontracture (559.0 ± 23.8 s, Ip + CsA, vs. 455.2 ± 15.3 s, Ip, $p<0.05$) due to ipratropium treatment alone.

5.3.7 Cyclosporin A mediated protection against ipratropium induced myocardial injury is associated with downregulation of phospho-Akt

Increased levels of phospho-Akt associated with ipratropium induced exacerbation of myocardial injury were reported in chapter 4. It was observed that the cardioprotective properties of acetylcholine were associated with a decrease in the levels of phospho-Akt, following 5, 15 and 120 minutes reperfusion. In this study, hearts were harvested 5, 15 and 120 minutes after the onset of reperfusion where ipratropium (1×10^{-7} M) ± CsA (2×10^{-7} M) were administered as reperfusion was initiated. Western blot analysis was used to determine the expression levels of phospho-Akt as a percentage of total Akt in the homogenised heart samples.

As shown in Figure 5.10, at all three time points, hearts subjected to 35 minutes ischaemia and ipratropium (1×10^{-7} M) administration at the onset of reperfusion showed a statistically significant increase in p-Akt in comparison with control I/R hearts for the corresponding time point (Values in Figure 5.10 (**D**), $p<0.01$ for all groups). In contrast to this, at all time points, there was a significant decrease in p-Akt levels following cyclosporin A administration in comparison with the respective controls (44.9

± 18.6 %, 5 mins, 55.7 ± 26.7 %, 15 mins and 49.8 ± 23.3 % vs. 100.0 ± 0.0 % (respective control), $p<0.01$ for all groups).

For the groups where CsA (2×10^{-7} M) and ipratropium (1×10^{-7} M) were co-administered at the onset of reperfusion, at all time points, there was an increase in p-Akt levels in comparison with both the control and treatment groups where CsA (2×10^{-7} M) was administered alone. At all time points, these were significant, as denoted in panel **D** in Figure 5.10. However, it was only at the 5 minute time point that there was a significant result in comparison with the ipratropium treatment group (183.4 ± 16.6 %, Ip + CsA, vs. 260.2 ± 36.1%, Ip, $p<0.05$). At the other time points, despite not being significant in comparison with the group treated with ipratropium alone, there was a statistical increase in p-Akt levels in comparison with the groups treated with CsA alone, as denoted in panel **D** of Figure 5.10.

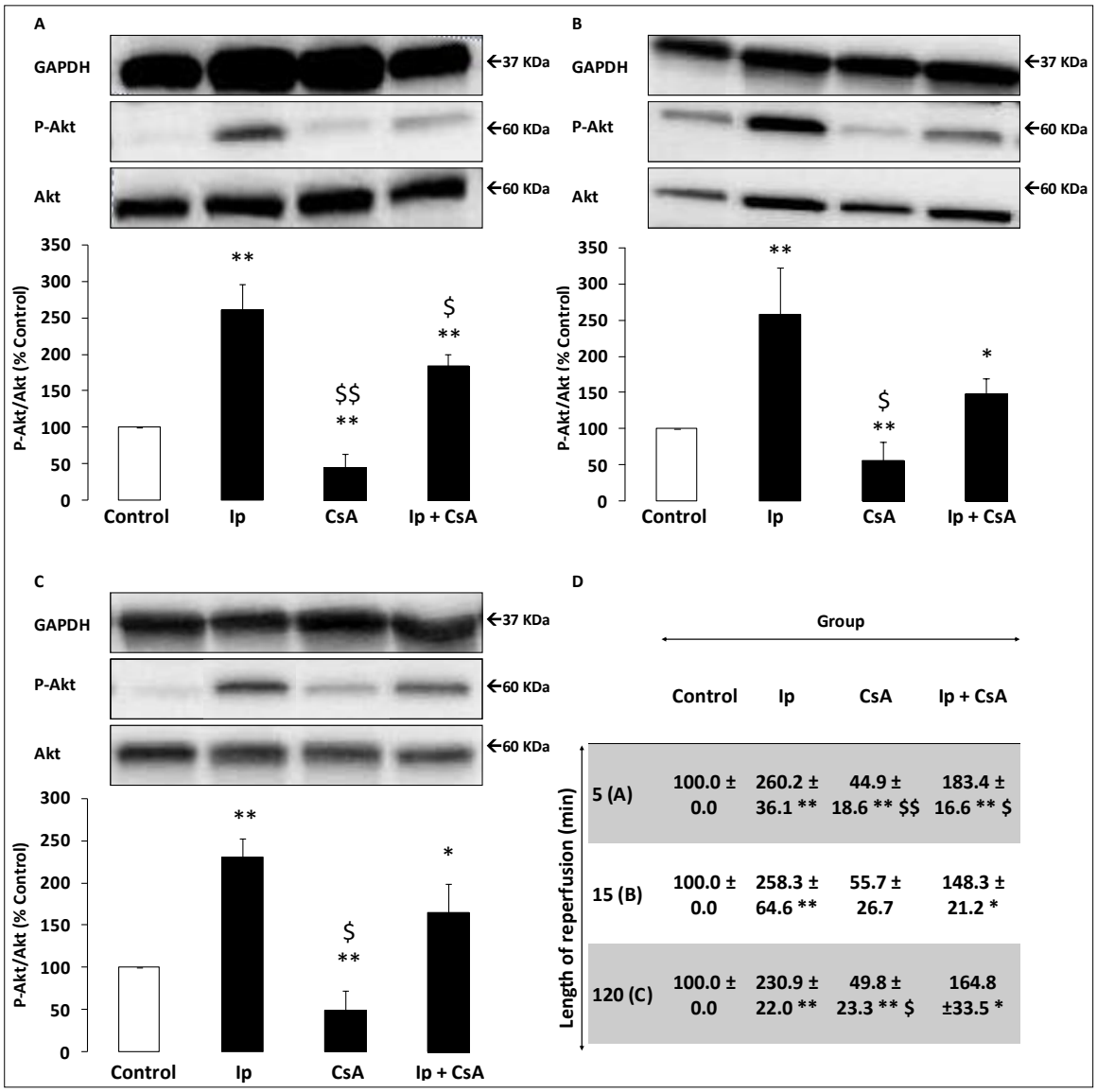


Figure 5.10 The effects of ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) treatment on the levels of phosphorylated Akt following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-Akt and total Akt for each time point shown above figures with treatment groups in the following order for all blots: Control, Ip, CsA and Ip + CsA. All values expressed as mean + SEM of p-Akt/Akt as a percentage of the control (**D**). * p<0.05 and ** p<0.01 vs. respective control, $ p<0.05 and $$ p<0.001 vs. respective Ip treated group, n=3 for all groups.

5.3.8 Effect of cyclosporin A and ipratropium on levels of phospho-Erk 1/2 following *in vitro* I/R myocardial injury

In chapter 4, following 5, 15 and 120 minutes reperfusion, ipratropium administration was associated with a statistical increase in levels of phospho-Erk1/2. This increase was abolished following acetylcholine treatment; thereby associating a reduction in phospho-Erk1/2 with the observed protective properties of acetylcholine against ipratropium induced myocardial injury. In the present study, hearts were harvested 5, 15 and 120 minutes after the onset of reperfusion where ipratropium (1×10^{-7} M) ± CsA (2×10^{-7} M) were administered as reperfusion was initiated, in order to elucidate whether CsA protects against ipratropium induced injury via a mechanism involving phospho-Erk1/2. Western blot analysis was used to determine the expression levels of phospho-Erk1/2 as a percentage of total Erk1/2 in homogenised heart samples.

Figure 5.11 presents the data from this study. A significant increase in levels of phospho-Erk1/2 was observed at all time points following ipratropium (1×10^{-7} M) administration at reperfusion in comparison with the untreated control groups (157.5 ± 24.6 %, 5 mins, 145.5 ± 27.2 %, 15 mins and 172.4 ± 21.3 %, 120 mins vs. 100.0 ± 0.0 % (respective control), $p < 0.01$ for all groups). Treatment with CsA alone caused a reduction in the levels of Erk1/2 phosphorylation at all time points as compared with the respective untreated control (58.8.5 ± 32.1 %, 5 mins, 61.7 ± 24.6 %, 15 mins and 43.4 ± 15.7 %, 120 mins vs. 100.0 ± 0.0 % (respective control), $p < 0.05$ for 5 and 15 mins groups, $p<0.01$ for 120 mins group). Interestingly, concomitant treatment of ipratropium and CsA caused significant increases in Erk1/2 phosphorylation in

comparison with the control, however these levels were statistically indifferent from the groups treated with ipratropium alone at all time points.

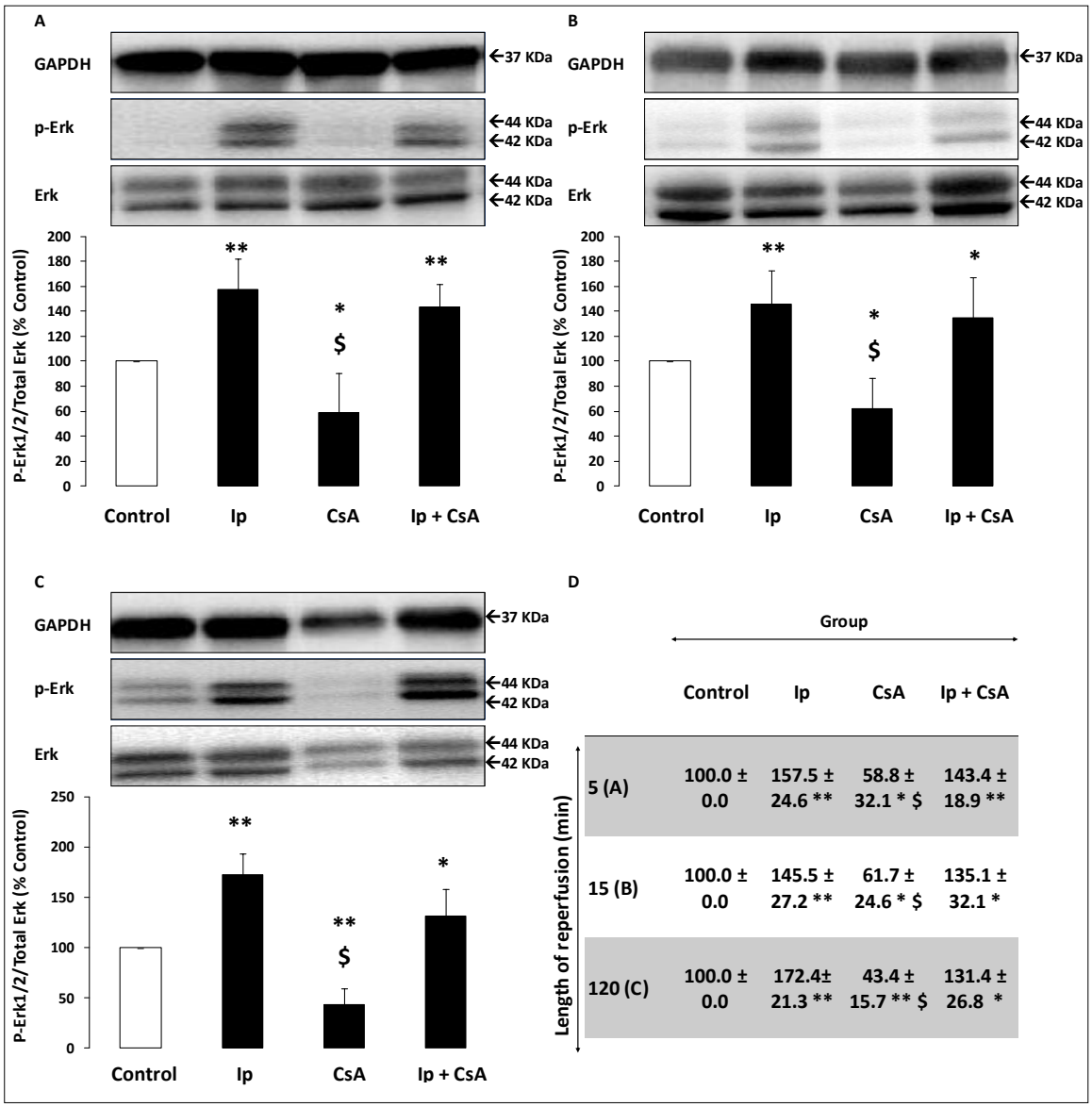


Figure 5.11 The effects of ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) treatment on the levels of phosphorylated Erk 1/2 following 35 minutes regional ischaemia and 5 **(A)**, 15 **(B)** and 120 **(C)** minutes reperfusion. Corresponding representative blots of GAPDH, p-Erk 1/2 and total Erk 1/2 for each time point shown above figures with treatment groups in the following order for all blots: Control, Ip, CsA and Ip + CsA. All values expressed as mean + SEM of p-Erk 1/2/Erk 1/2 as a percentage of the control **(D)**. * $p<0.05$ and ** $p<0.01$ vs. respective control $ $p<0.05$ vs. Ip, n=3 for all groups.

5.3.9 Cyclosporin A mediated protection against ipratropium induced myocardial injury is not associated with phosphorylation of JNK

It was previously shown (in Chapter 4), following 5, 15 and 120 minutes reperfusion, ipratropium (1 x 10^{-7} M) administration was only associated with a statistical difference in phospho-JNK in comparison with the untreated control following 15 minutes reperfusion (66.7 ± 20.2%, Ip, vs. 100.0 ± 0.0%, control, $p<0.05$). JNK has been shown to initiate apoptosis via phosphorylation of BAD. However, significant decreases in phospho-BAD levels were observed, via flow cytometry, following ipratropium (1 x 10^{-7} M) administration. As CsA protects via mitochondrial mechanisms which regulate both apoptosis and necrosis, this study aimed to identify whether CsA administration had an effect on the phosphorylation of BAD and, if this would provide additional information as to the mechanism by which CsA abrogates ipratropium induced myocardial injury.

The results of the Western blots are presented in Figure 5.12. As previously described, tissue was harvested following 5, 15 and 120 mins reperfusion where ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) were administered as reperfusion was initiated. Although CsA (2 x 10^{-7} M) showed increases in JNK phosphorylation (when administered alone and co-administered with ipratropium) in comparison with the ipratropium treated groups, at all time points investigated, these were statistically insignificant in comparison with the respective ipratropium (1 x 10^{-7} M) treated groups. In comparison with the respective control groups, none of the groups treated with CsA (either alone or in conjunction with ipratropium) showed significant differences in phospho-JNK levels.

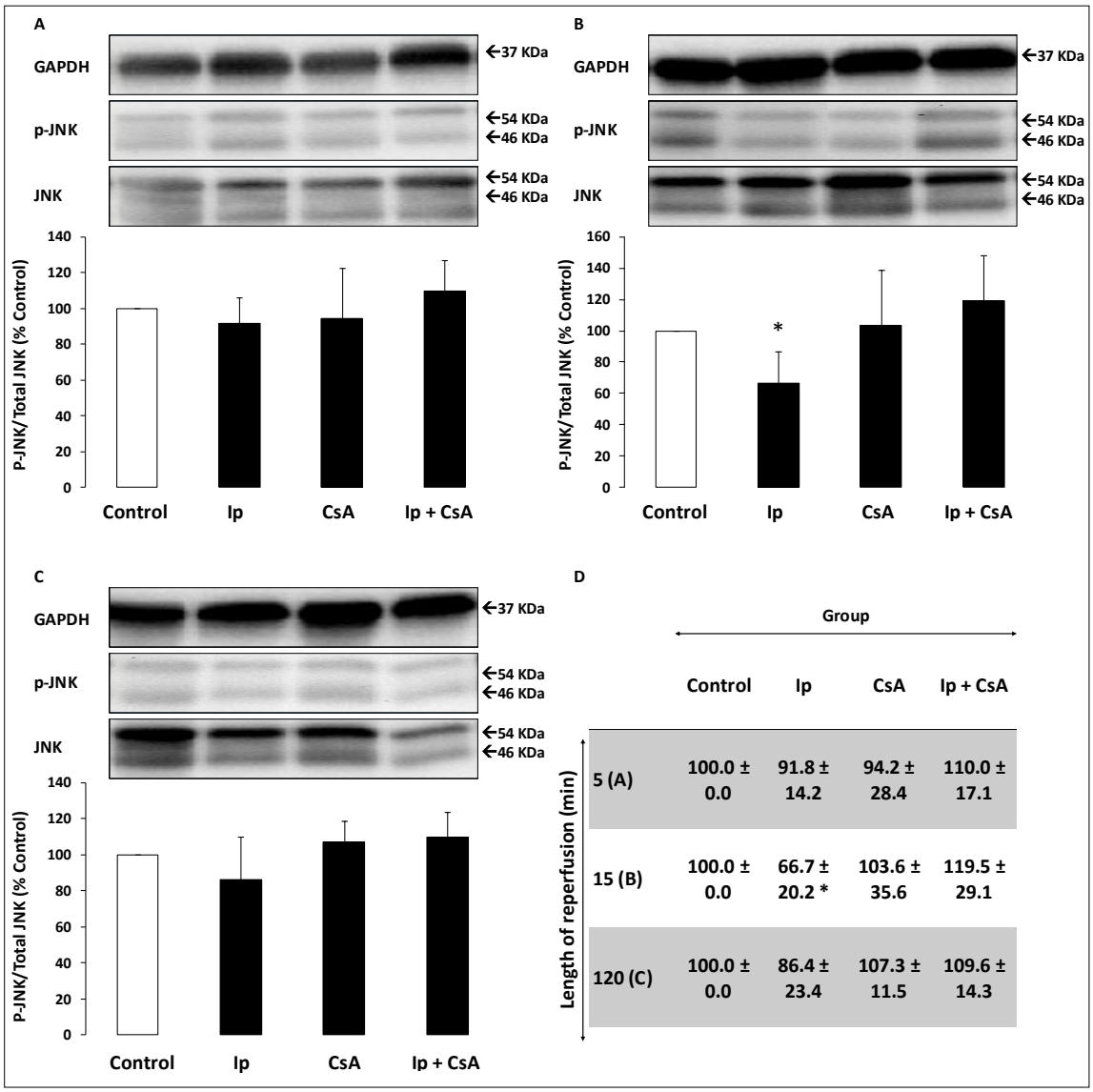


Figure 5.12 The effects of ipratropium (1 x 10^{-7} M) ± CsA (2 x 10^{-7} M) treatment on the levels of phosphorylated SAPK/JNK following 35 minutes regional ischaemia and 5 (**A**), 15 (**B**) and 120 (**C**) minutes reperfusion. Corresponding representative blots of GAPDH, p-SAPK/JNK and total SAPK/JNK for each time point shown above figures with treatment groups in the following order for all blots: Control, Ip, CsA and Ip + CsA. All values expressed as mean + SEM of p-JNK/total JNK as a percentage of the control (**D**). * $p<0.05$ vs. untreated control (15 mins), n=3 for all groups.

5.4 Chapter Discussion

In this study, it was determined that CsA protects the myocardium following an ischaemic insult, when administered at the onset of reperfusion or re-oxygenation in the isolated perfused rat heart and primary cardiac myocytes. As CsA is known to be cardioprotective via its inhibition of mPTP opening, these results were expected and verified the experimental techniques used.

It was also demonstrated that CsA also protects the myocardium from ipratropium induced cardiac injury following ischaemia/reperfusion, when administered at the onset of reperfusion. This is shown by a reduction in infarct size and myocyte viability in comparison with the groups treated with ipratropium (1×10^{-7} M) alone in the Langendorff model and MTT assay. CsA is known to exert its protective effect by blocking mPTP opening via interaction with Cyclophilin D, a key component of the mPTP (Basso et al. 2005, Schinzel et al. 2005), situated in the inner mitochondrial membrane (Hausenloy et al. 2010). The ability for CsA to partially reverse the observed ipratropium induced toxicity, in these two models, therefore indicates a role for the mitochondria in ipratropium induced myocardial injury.

Interestingly, as shown by flow cytometry, administration of CsA in conjunction with ipratropium was capable of reducing overall myocyte loss, but only significantly compensated for loss of myocytes by necrosis rather than caspase mediated apoptosis. Further to this, there were no observed changes in levels of the cleaved caspase-3 or phospho-BAD in comparison with the untreated controls in the groups treated with CsA alone, indicating that intrinsic apoptosis does not play a role in CsA induced

cardioprotection. In line with this, CsA, when co-administered with ipratropium, was not capable of significantly attenuating the increased levels of cleaved caspase-3 or phospho-BAD associated with ipratropium treatment. However, as observed by the Langendorff model and MTT assay, CsA is capable of eliciting significant protection against ipratropium induced myocardial injury. This indicates that CsA exerts its protective effect, primarily, via prevention of necrosis. These data promote the hypothesis that ipratropium induced injury has both apoptotic and necrotic components, and that the ability for CsA to protect against the necrotic component is sufficient to significantly compensate for ipratropium induced injury.

The mPTP studies support mitochondrial involvement more comprehensively, as in this model depolarisation is indicative of mPTP opening and hypercontracutre occurs as subsequent cell death from associated ATP depletion. The ability of ipratropium treatment to drecease the time for both depolarisation (indicative of the mPTP opening) and hypercontracture indicates that ipratropium exerts a toxic effect via disruption of the mPTP. This is further supported by the ability of CsA to compensate for the ipratropium induced reduction in these times, thereby protecting myocytes against ipratropium induced injury following oxidative stress.

It was previously shown that the administration of ipratropium in non-ischaemic and in ischaemic, but with the absence of reperfusion, models (as shown in Chapter 3) has no toxic effect in comparison with untreated controls. This indicates that ipratropium specifically exacerbates reperfusion injury rather than exerting a generalised toxic effect on the heart. It has been documented that the mPTP opens at reperfusion (Griffiths and Halestrap 1995), whereas it remains closed during ischaemia. Further to

this the mPTP plays a central role in mediating reperfusion induced damage to the myocardium (Yellon and Hausenloy 2007b) as opening of the mPTP determines the fate of myocytes. The mPTP was previously considered to comprise of ANT (Adenine Nucleotide Transferase), VDAC (Voltage Dependent Anion Channel) and Cyclophilin-D, with a role for the mitochondrial phosphate carrier. However, recently it has been elucidated that ANT and VDAC do not appear to be critically involved in forming the channel of the mPTP (Baines 2009) as genetic ablation of both ANT and VDAC do not prevent mPTP opening (Miura and Tanno 2011). This gives rise to the hypothesis that although Cyclophilin D is involved as a critical regulatory component of the mPTP, it is not structurally involved (Baines 2009, Elrod et al. 2010).

Despite the fact the components of the mPTP remain as a matter of some contention, it is known that initiation of reperfusion following a period of ischaemia, causes opening of the mPTP. The mPTP mediates the increase in mitochondrial permeability. When the non-specific mPTP opens, it permits passage of molecules ≤1.5 KDa (66 Yellon,Derek M. 2007; 61 Halestrap,A.P. 2009; 367 Pasdois,P. 2011), leading to increased osmotic pressure on the mitochondrial membrane causing subsequent mitochondrial swelling and outer mitochondrial membrane rupture. This is associated with the initiation of cell death, both by apoptosis, via movement of cytochrome c into the cytosol, however, necrotic death also occurs via collapse of the mitochondrial membrane potential, ultimately leading to ATP depletion (Hausenloy and Yellon 2004) accompanied by disruption of ionic and metabolic homeostasis. This study indicates that CsA protects primarily by a mechanism which prevents necrosis rather than caspase-3 mediated apoptosis.

Alongside prevention of mPTP opening, the activation of the reperfusion injury salvage kinase (RISK) pathway is also a main target for cardioprotection (Hausenloy and Yellon 2004), The pro-survival RISK pathway consists of the kinase signalling cascades, phosphatidylinositol-3-OH kinase (PI(3)K)-Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2) (Davidson et al. 2006). RISK pathway activation is necessary for protection by delaying opening of the mPTP (Davidson et al. 2006). Multiple, although not all, G-protein-coupled-receptor, pro-survival signalling pathways appear to target the mPTP (Miura and Tanno 2011). The use of acetylcholine has been shown to potently protect against reperfusion injury in both pre- (Critz, Cohen and Downey 2005, Qin, Downey and Cohen 2003) and post-(Xiong et al. 2009, Zang, Sun and Yu 2007) conditioning and, there has since been categorical links to the RISK pathway, as many of the components involved (including PI3K and Erk1/2) in acetylcholine induced protection are necessary players in the RISK pathway (Qin, Downey and Cohen 2003, Zang, Sun and Yu 2007).

It is possible that the antagonist action of ipratropium on muscarinic receptors in the heart may cause down regulation of PI3K and Erk1/2 and may, therefore, deregulate RISK pathway signalling which may induce pore opening at the mPTP level. In Chapter 4, it was demonstrated that ipratropium administration actually produced a significant increase in levels of phosphorylated Akt and Erk1/2. This supports previous work which associates increased Akt phosphorylation with exacerbation of myocardial injury following ischaemia/reperfusion due to the formation of reactive oxygen species and an increase in oxidative stress (Ushio-Fukai et al. 1999). In this study, it is postulated that the observed increases in phospho-Erk1/2 were as a consequence of increased

Akt levels. The current study provided evidence that CsA is capable of eliciting protection from ipratropium induced myocardial injury, and this is associated with relative down-regulation of Akt and Erk1/2 phosphorylation. It is interesting; however, that CsA was capable of significantly decreasing phospho-Akt levels when co-administered with ipratropium (in comparison with the ipratropium group) whereas CsA co-administration had no statistical effect on levels of phospho-Erk1/2. Both Akt and Erk1/2 serve as upstream regulators of apoptosis, however, Erk1/2 activation is involved in caspase-dependent apoptosis whereas Akt phosphorylation plays a larger role in caspase-independent apoptosis (Hausenloy 2004). There was no observed significant difference in cleaved caspase-3 when ipratropium and CsA were administered concurrently in comparison with the group treated with ipratropium alone. The Western blot analysis supports these results, as CsA is also incapable of eliciting a significant difference in phospho-Erk1/2 in comparison with the group treated with ipratropium alone, however statistically attenuated Akt levels.

Interestingly, it has been previously shown that, in the context of doxorubicin myocardial injury, in the context of I/R, that Akt and Erk1/2 levels are significantly decreased following CsA administration. In this study, CsA was capable of partially abrogating doxyrubicin injury (Gharanei et al. 2013). Despite major differences in the clinical application of ipratropium and doxyrubicin, doxyrubicin has been shown to elicit anti-muscarinic properties (Sasaki et al. 2010). It is therefore possible that the mode of action by which CsA protects against doxyrubicin injury is similar to that by which CsA protects against ipratropium induced myocardial injury following I/R.

Further to this, following I/R, Erk1/2 and Akt have been shown to induce necrosis when over expressed. In particular, this involves the action of p53 in initiating mitochondrial permeability transition, therby leading to mPTP opening and necrosis (Vaseva et al. 2012). Also, constitutive over expression of Erk1/2 is capable of coupling towards cellular death mechanisms (Xie and Yu 2007).

Given the evidence that CsA co-administration with ipratropium limits myocardial injury in comparison with ipratropium administration alone and that BAD is phosphorylated by JNK activation (Yu et al 2004), Western blot analysis for JNK was also conducted. There were no observed differences in levels of phospho-JNK in comparison with the respective ipratropium treated groups or untreated controls with groups treated with CsA (either alone or concomitantly with ipratropium). It has previously been demonstrated that dephosphorylation of BAD promotes apoptosis as it enables formation of Bak/Bax channels, thereby permitting release of pro-apoptotic factors such as cytochrome C and APAF-1 into the cytosol (Murriel et al 2004). This further supports the accumulating evidence that CsA does not protect against ipratropium induced myocardial injury through an apoptotic mechanism.

Despite much controversy, it was previously assumed that the mPTP and Bax/Bak channels may be structurally linked, or, indeed, consist of the same components (Karch et al 2013). This assumes that myocyte loss following ischaemia/reperfusion and mPTP opening would be attributed to apoptosis. It has, however, recently been elucidated that, although Bax and Bak are, in context, responsible for intrinsic apoptosis, loss of Bax/Bak limits function of the mPTP. Further to this, Bax has subsequently been shown to regulate necrosis via its association (potentially as the outer mitochondrial

membrane component of) the mPTP (Whelan et al. 2012). Given the evidence that CsA co-administration with ipratropium limits myocardial injury via a mechanism which limits necrosis rather than apoptosis, it is postulated that, even though the mPTP may consist of regulatory proteins which regulate apoptosis, they may also have a secondary role whereby CsA can protect via necrosis.

In this study, it has been established that CsA protects against ipratropium induced myocardial injury following ischaemia reperfusion via a protective mechanism which involves the mPTP and regulation of the RISK pathway proteins, Akt and Erk1/2.

Chapter 6 Ipratropium further exacerbates myocardial ischaemia reperfusion in the aged rat myocardium

6.1 Chapter introduction and purpose

Individuals who are over 60 years of age are classified as elderly and it is currently estimated that within the next decade, over 20% of total global population will classify as the "elderly population" (Liu et al. 2002). In addition to this, the average age for a chronic obstructive pulmonary disease (COPD) diagnosis is 67 years and over 80% of patients with ischaemic heart disease (IHD) are over 65 years of age (Rosamond et al. 2008).

Despite the knowledge that myocardial infarction is more common in the elderly (Valente et al. 2010) and mounting evidence that myocardial I/R injury is more severe, as shown both clinically and experimentally (Lesnefsky et al. 1994, Lesnefsky et al. 2003, Kajstura et al. 1996, Boengler et al. 2009), the cellular mechanisms responsible are yet to be fully elucidated. However, despite this, there is a significant lack of literature concentrating on the physiological mechanisms involved in I/R in the aged heart.

From the perspective of anti-cholinergics, there is controversial evidence that elderly COPD patients require different pharmacological therapies in comparison with younger patients with respiratory diseases (Valente et al. 2010). However, although clinical trials have not identified any age-associated loss in the efficacy of anti-cholinergics, such as ipratropium (Valente et al. 2010), there are no clinical or pre-clinical studies to

address whether ipratropium, or other anti-cholinergic compounds, are associated with increased myocardial injury. In particular, due to the frequent concurrence of IHD and COPD, and with the knowledge that COPD patients are at a high risk of myocardial infarction, there is a distinct lack of research to identify whether anti-cholinergics are capable of exacerbating myocardial ischaemia/reperfusion injury in the senescent heart.

Within the respiratory and cardiovascular systems, the primary subtypes of muscarinic receptors present are M_2 and M_3 (Haddad et al. 1999, Wessler and Kirkpatrick 2008). The antagonistic action of ipratropium at the M_3 receptors in the airways is responsible for the alleviation of bronchoconstriction, thereby facilitating airflow and relieving COPD exacerbations (Barnes 2004). The agonistic action of acetylcholine within the myocardium has been shown as instrumental in limiting infarct size following I/R injury via a mechanism which activates the reperfusion injury salvage kinase (RISK) pathway and preservation of mitochondrial integrity through prevention of mitochondrial transition pore (mPTP) opening (Sun et al. 2010).

There is accumulating evidence that aging is associated with a reduction in functional expression of the M_2 (the subtype which accounts for the majority of mAChRs by density in the myocardium) (Abrams et al. 2006). The consequence of this is that endogenous acetylcholine is incapable of eliciting the negative inotropic and chronotropic effects observed in younger individuals (Brodde et al. 1998). However, it has not been ascertained whether the cardioprotective properties of acetylcholine are abolished in the aged myocardium. Under conditions of I/R, muscarinic pro-survival

signalling is instrumental in limiting infarct size, prevention of mPTP opening and promoting activation of the RISK pathway (Liu et al. 2013, Sun et al. 2010a).

6.2 Methods

21 adult (3 month), 21 12 month, 21 18 month and 15 24 month male, Sprague-Dawley rats were used throught the experiments detailed in this chapter. All experiments were subject to the exclusion criteria detailed in Chapter 2, section 2.8. For all data, results are presented a group means + standard error of the mean (SEM). For the Western blotting data, values have been normalised to the respective untreated control, which is represented as 100%. Statistical differences were ascertained with the use of one-way ANOVA, with the exception of haemodynamics which where a two-way ANOVA was employed, and Fisher's LSD post-hoc test.

6.2.1 Isolated perfused rat heart model

Following sacrifice by cervical dislocation and excision, hearts from 3, 12, 18 and 24 month old rats were mounted on Langendorff perfusion apparatus. For all experiments, the Langendorff model of I/R was used. Ipratropium (1×10^{-7} M) was administered after 55 mins, at the onset of reperfusion. At the end of the experimental protocol, infarct size to risk ratio (I/R %) was determined via Evans blue and TTC staining. Throughout all experiments, left ventricular developed pressure (LVDP, mmHg), heart rate (HR, beats.min^{-1}) and coronary flow (CF, ml.min^{-1}) were measured to assess the stability of the hearts.

6.2.2 mPTP model of oxidative stress

Cardiac myocytes were isolated as described in Chapter 2, section 2.6. Following adhesion to laminin coated cover slips, adhered myocytes from 3, 12 and 18 month old rats were incubated in microscopy buffer containing 3 x 10^{-6} M, tetramethylrhodamine methyl ester (TMRM) for 15 minutes. Cells were washed and randomly assigned to one of the following groups: untreated control, Ip (1 x 10^{-7} M), ACh (1 x 10^{-7} M) CsA (2 x 10^{-7} M), Ip + ACh or Ip + CsA (concentrations as before). Myocytes were viewed and analysed via confocal microscopy. Depolarisation (Dep) was measured as the time at which the TMRM started to become evenly distributed throughout the cell and is indicative of the initiation of the mPTP opening. Subsequent hypercontracture (Hyp) of myocytes occurs shortly afterwards due to ATP depletion. The time to both Dep and Hyp were recorded.

6.2.3 Western blotting

Ventricular tissue was collected via Langendorff perfusion, following the protocol in Chapter 2, sections 2.6.1 and 2.6.2. Tissue was harvested following 5, 15 and 120 min reperfusion, as previously described, from 3, 12, 18 and 24 month old rats. Ipratropium (1 x 10^{-7} M) was administered at the onset of reperfusion. Following homogenisation and storage in suspension buffer, 60 µg of protein from was diluted with sample buffer and loaded into precast gels from samples of the following groups: untreated I/R control or ipratropium (1 x 10^{-7} M). After separation by electrophoresis, proteins were transferred onto PVDF membranes and probed for the phosphorylated forms of Akt, Erk1/2 and JNK. To assess relative changes in protein levels, membranes were

subsequently probed for the total, unphosphorylated forms of Akt, Erk1/2 and JNK and relative changes in phosphorylated protein were calculated as a percentage of total protein level. To ensure protein loading was equal, all membranes were also probed for GAPDH.

6.3 Results

6.3.1 The observed exacerbation of myocardial ischaemia/reperfusion injury following ipratropium administration is more severe in the aged myocardium

In order to ascertain whether ipratropium also elicits a cardiotoxic effect following I/R in the aged myocardium, isolated Langendorff heart experiments were conducted on 3, 12, 18 and 24 month old Sprague Dawley rat hearts. In all different age control groups, hearts were subjected to the I/R protocol (as previously described, section 2.3.4), in the ipratropium treatment groups, ipratropium (1×10^{-7} M) was administered at the onset of reperfusion.

6.3.1.1 Effect of aging and ipratropium treatment on infarct development following ischaemia/reperfusion

The results presented in Figure 6.1 show the effect of aging on infarct development in isolated perfused Langendorff rat hearts. In the 12, 18 and 24 month control groups, infarct development (I/R%) was statistically increased in comparison with the 3 month control group ($p<0.01$ for all groups, values in table 6.1). However, there was no statistical difference in infarct size in comparison with the 12, 18 or 24 month age groups.

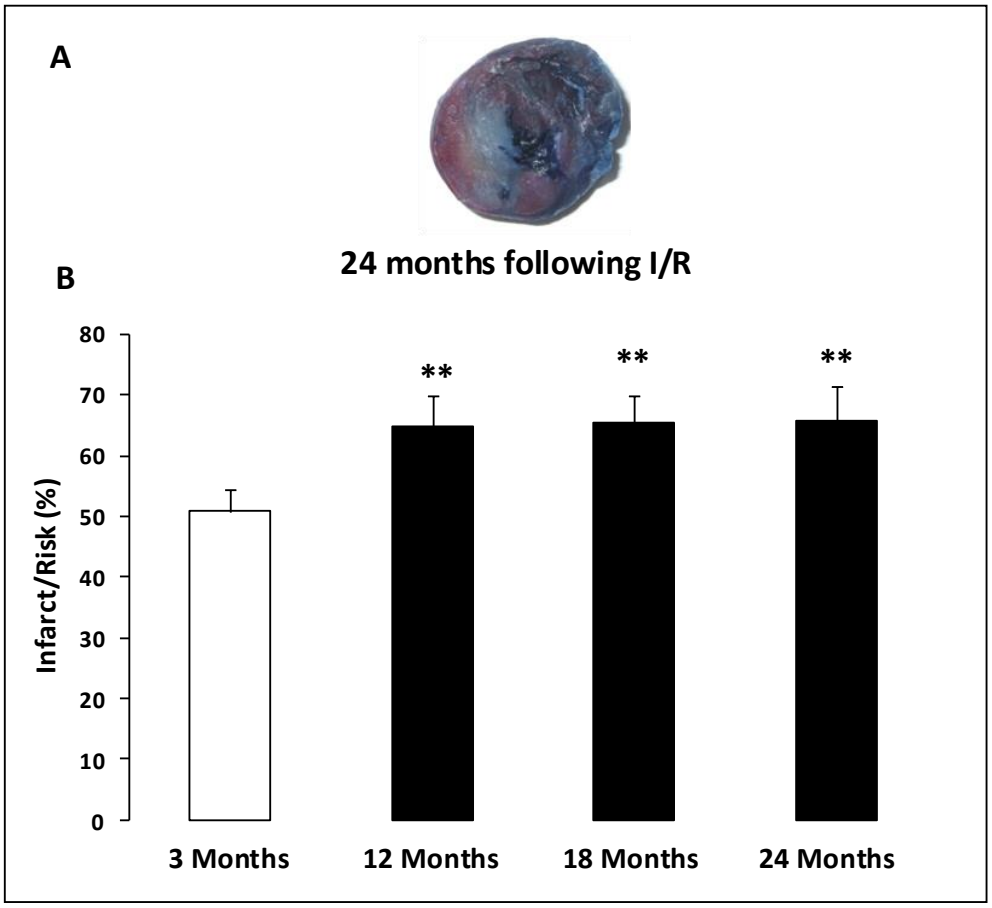


Figure 6.1: A. Representative heart slices showing Evans blue and TTC staining following I/R protocol. Viable tissue is delineated as blue, risk tissue as red and infarction as grey/white. **B**. I/R% of 3, 12, 18 and 24 month Langendorff hearts following I/R protocol. ** $p<0.01$ vs. 3 month group, n=3.

Age	3 months		12 months		18 months		24 months	
	Control	Ip	Control	Ip	Control	Ip	Control	Ip
Infarct/Risk (%)	51.8 ± 3.0	75.1 ± 4.3	64.8 ± 5.3	74.9 ± 5.4	65.4 ± 4.7	84.6 ± 6.3	65.8 ± 5.6	95.8 ± 9.3

Table 6.1: Infarct development in the risk zone following ipratropium administration in Sprague Dawley rats ages 3, 12, 18 and 24 months.

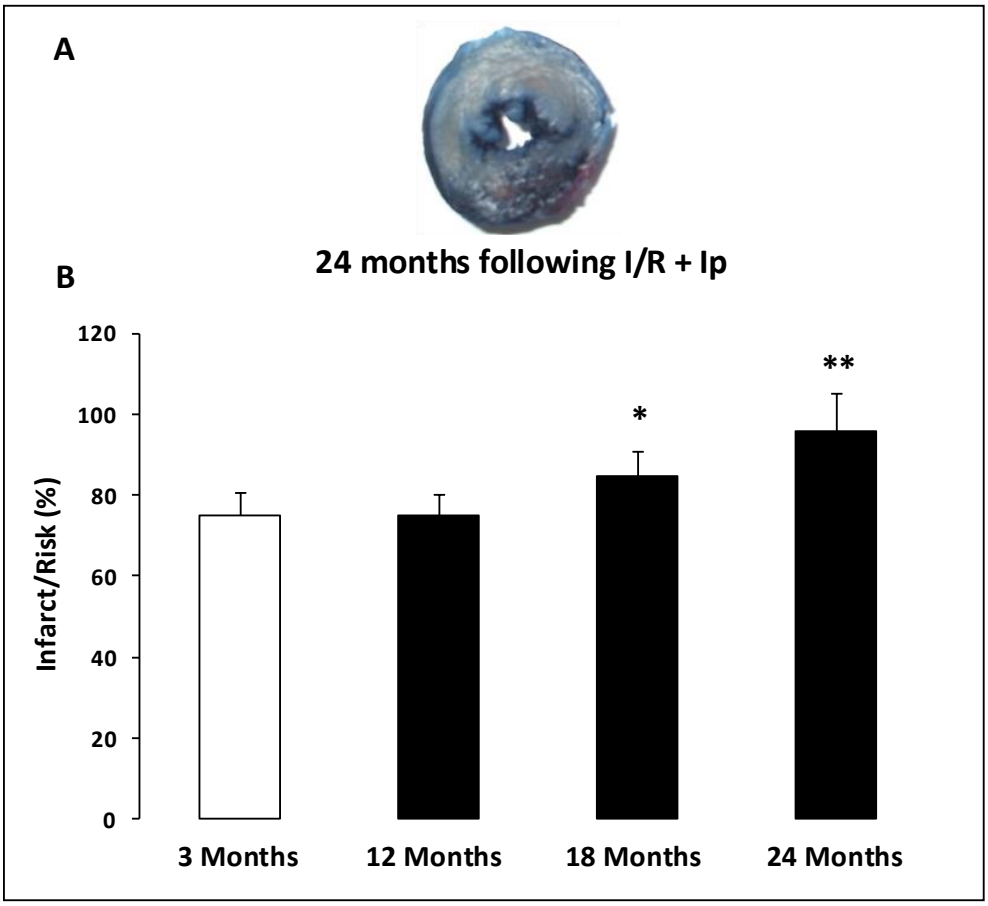


Figure 6.2: A. Representative heart slices showing Evans blue and TTC staining following I/R protocol in the presence of ipratropium (1 x 10^{-7}M) administration. Viable tissue is delineated as blue, risk tissue as red and infarction as grey/white. **B**. I/R% of 3, 12, 18 and 24 month Langendorff hearts following I/R protocol and 1 x 10^{-7} M ipratropium administration at reperfusion.* $p<0.05$ and ** $p<0.01$ vs. 3 month group, n=3.

Following aging, in the groups treated with ipratropium (1 x 10^{-7} M), there was an apparent age responsive increase in I/R % compared with the 3 month ipratropium treatment group. The results are presented in Figure 6.2 show the effect of aging and ipratropium administration on infarct development in isolated perfused Langendorff rat hearts. With the exception of the 12 month age group, ipratropium administration significantly increased I/R% in comparison with the 3 month, ipratropium treated, group (*$p<0.05$, **$p<0.01$ vs. 3 months, Figure 6.2, values in Table 6.1).

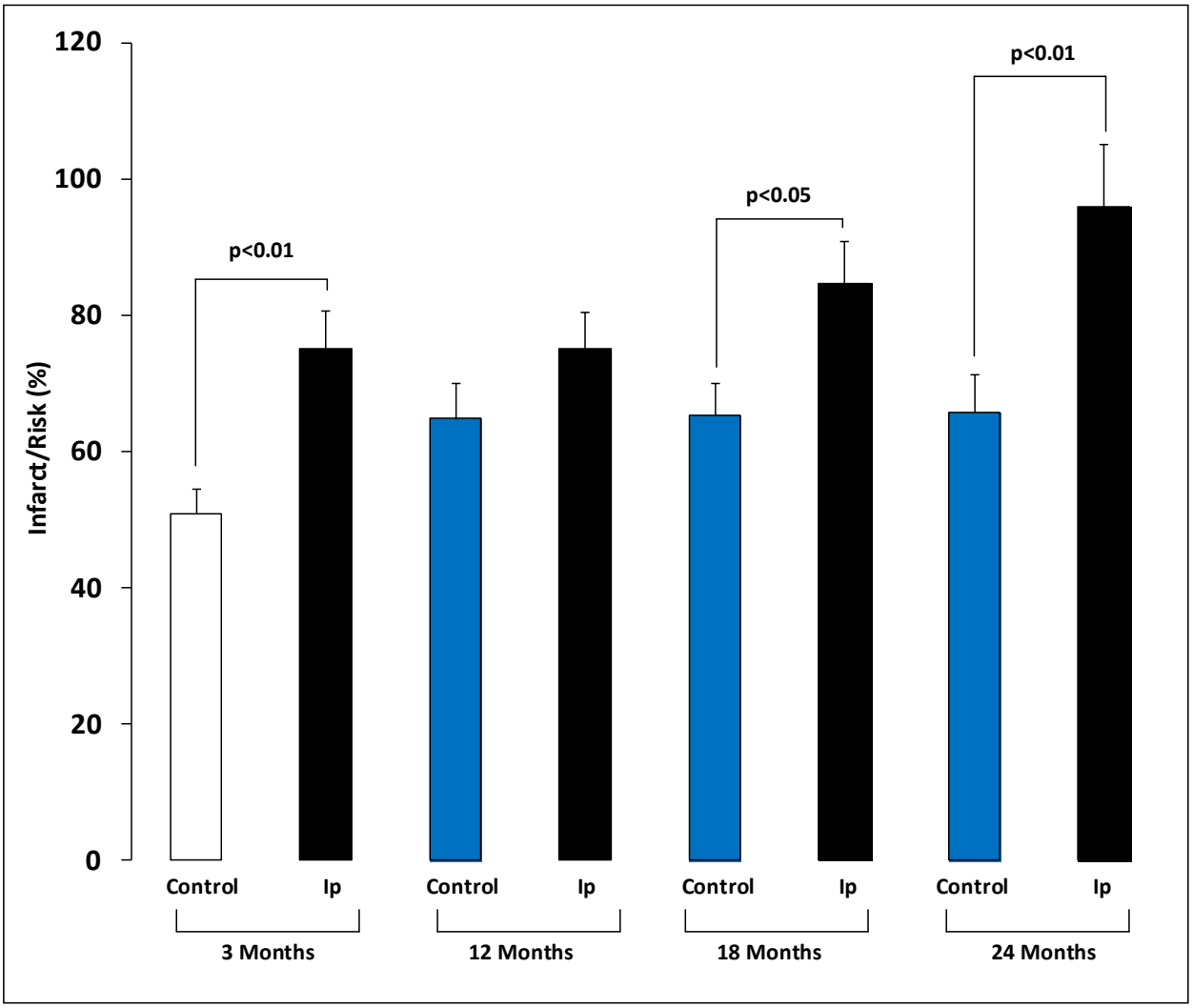


Figure 6.3: Infarct development in the risk zone following ipratropium (1 x 10^{-7} M) treatment in isolated perfused rat heart from rats of 3, 12, 18 and 24 months of age.Infarct to risk ratio following ipratropium administration at the onset of reperfusion. Results are presented as infarct/risk (%).* p<0.05 vs. 3 months control, #p<0.05 vs. 3 months ipratropium treated group. Results expressed as mean + SEM, n=6.

With the exception of the 12 month age group, ipratropium administration significantly increased I/R% in comparison with the respective control (untreated) group from the same age group (statistical values represented on Figure 6.3, values in Table 6.1).

6.3.1.2 Haemodynamic parameters

All haemodynamic parameters were measured and expressed as previously described (section 2.3.3), and are presented in Figures 6.5 (LVDP), 6.6 (HR) and 6.7 (CF). There were no observed statistical differences in LVDP, HR or CF in comparison with hearts in the control group with any treatment group (ipratropium (Ip, $1x10^{-7}$ M), CsA ($2x10^{-7}$ M) or Ip + CsA) at any time point throughout the experimental procedure.

6.3.1.3 Left ventricular developed pressure (LVDP)

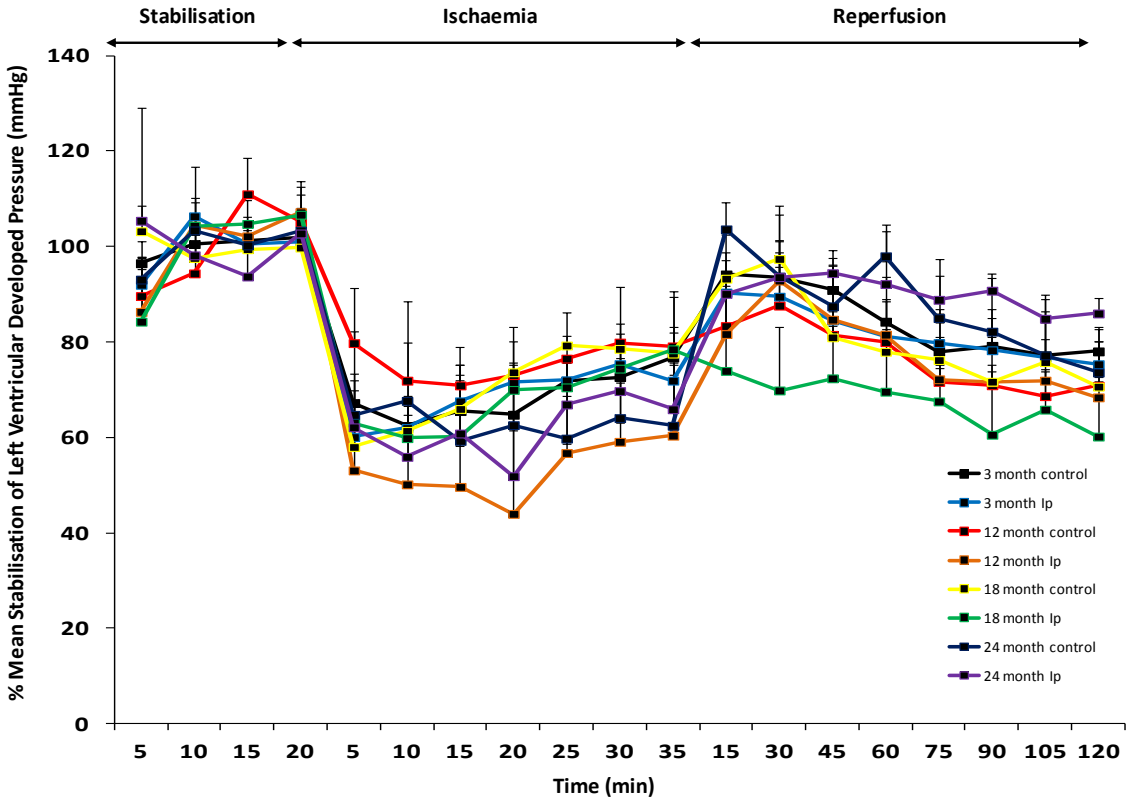


Figure 6.4: Changes in left ventricular developed pressure in isolated perfused rat hearts from 3, 12, 18 and 24 month old rats subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium (1×10^{-7} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 3 for all groups ($p>0.05$).

6.3.1.4 Heart rate (HR)

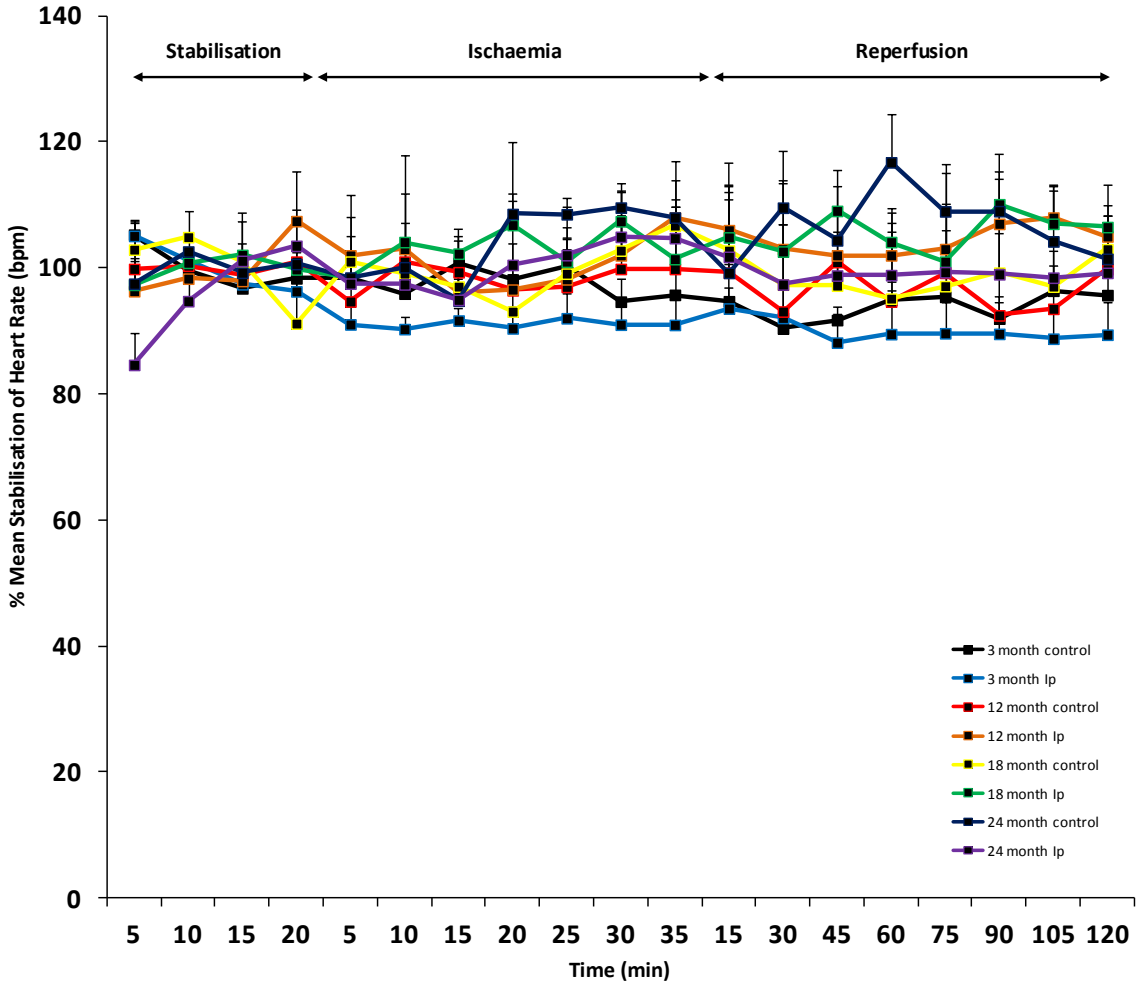


Figure 6.5: Changes in heart rate in isolated perfused rat hearts from 3, 12, 18 and 24 month old rats subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium (1×10^{-7} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 3 for all groups ($p>0.05$).

6.3.1.5 Coronary flow (CF)

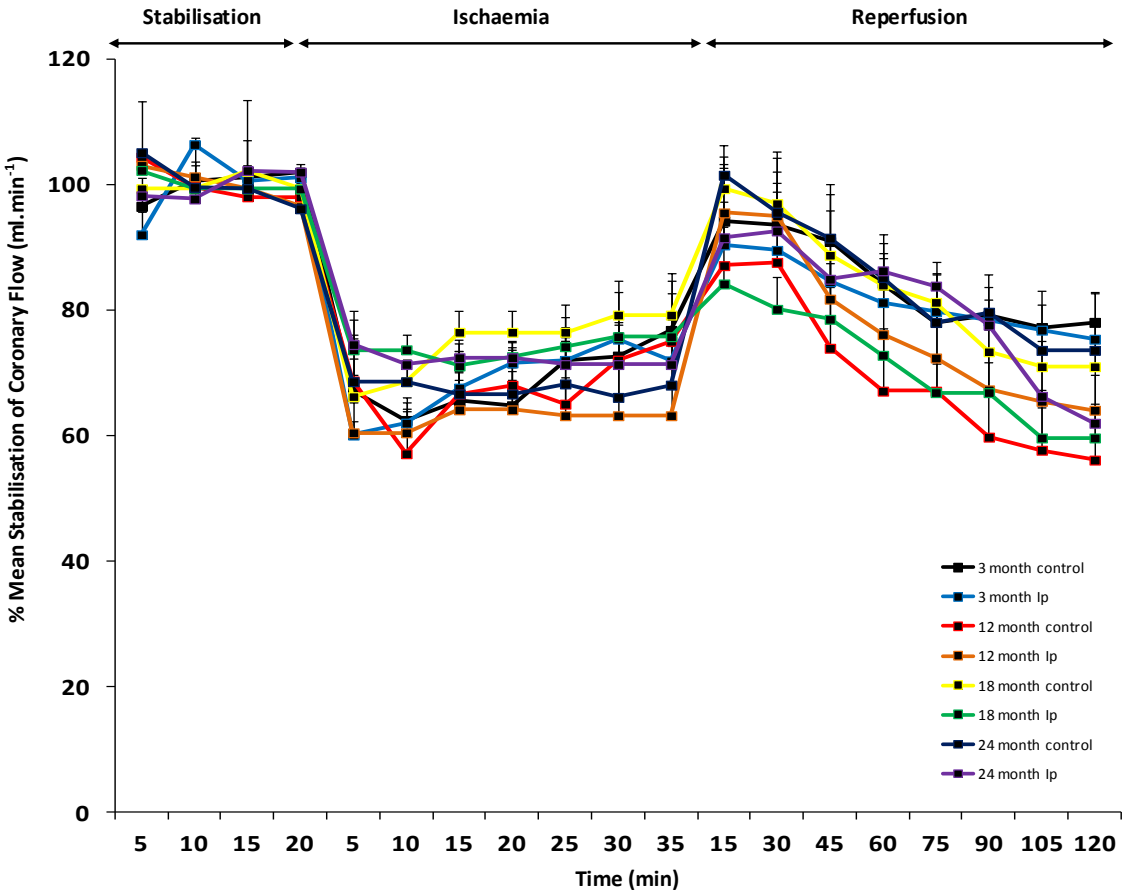


Figure 6.6: Changes in coronary flow in isolated perfused rat hearts from 3, 12, 18 and 24 month old rats subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion. Ipratropium (1 x 10^{-7} M) was administered at the onset of, and throughout, reperfusion. Values expressed as mean percentage of the stabilisation period + SEM, n= 3 for all groups ($p>0.05$).

6.3.2 The role of mPTP opening in aged rat hearts following ipratropium ± acetylcholine administration in a model of oxidative stress

As previously shown in Chapter 4, administration of acetylcholine (1 x 10^{-7} M) was shown to be capable of partially reversing ipratropium induced myocardial injury following I/R injury (as observed in the isolated perfused rat heart model) and significantly protecting against the loss of mitochondrial membrane integrity in the isolated myocyte model of oxidative stress. This is supported by previous studies where acetylcholine has been shown to be cardio-protective, as well as instrumental in pre- and post-conditioning, via a mechanism involving activation of the RISK pathway and prevention of mPTP opening (Sun et al. 2010a).

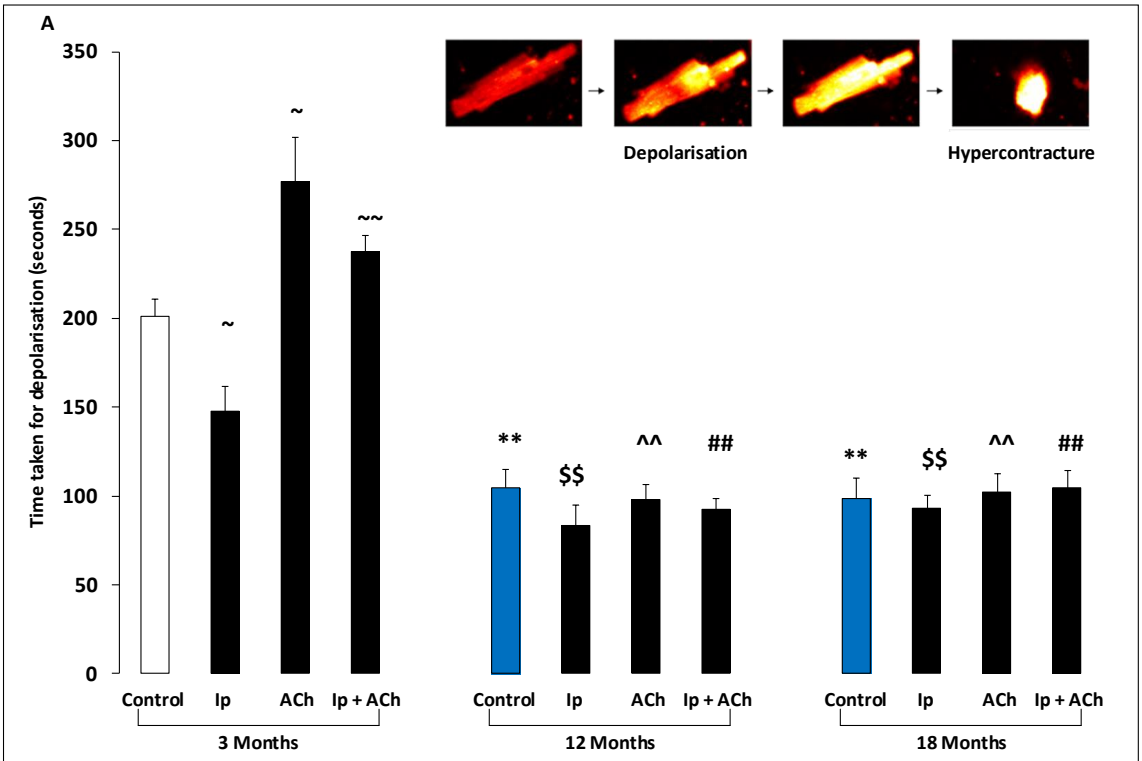
In the present study, the model of oxidative stress was employed to identify whether acetylcholine was capable of eliciting protection in this model and also whether acetylcholine could protect against ipratropium induced myocardial I/R injury in the aged myocardium. In order to address this, 3, 12 and 18 month old male Sprague Dawley rats were used. The model of oxidative stress was carried out using ipratropium (1 x 10^{-7} M) ± acetylcholine (1 x 10^{-7} M) and, as previously described, the time taken to depolarisation and hypercontracture were measured via use of confocal microscopy.

Figure 6.7 presents the results, for depolarisation, from the oxidative stress protocol. The main observation is that the time to depolarisation in the controls from both the 12 and 18 month old rats were significantly decreased in comparison with the time to depolarisation in the 3 month control (104.8 ± 10.6 s and 98.5 ± 11.8 s, 12 and 18

months respectively vs. 201.2 ± 9.9 s 3 months, $p<0.01$). As observed, in the 3 month age group, ipratropium (1 x 10^{-7} M) significantly decreased the time to depolarisation in comparison with the untreated control (147.5 ± 17.5 s, Ip, $p<0.01$). In comparison with this, the time to depolarisation was significantly increased following acetylcholine (1 x 10^{-7} M) administration, in both the presence and absence of ipratropium (276.7 ± 25.6 s, ACh, and 237.4 ± 9.3 s, Ip + ACh, $p<0.05$ and $p<0.01$ respectively). In contrast to this, in the 12 and 18 month age groups, there was no significant change in the time to depolarisation following any of the drug treatment groups (Ip (1 x 10^{-7} M), ACh (1 x 10^{-7} M) or Ip + ACh (both 1 x 10^{-7} M)) in comparison with the respective control group. However, in 12 and 18 month old rats, all treatment groups showed a significant decrease in time to depolarisation in comparison with the respective treatment group in 3 month old rats, values in table 6.2.

	Time taken to depolarisation (secs)		
	3 months	**12 months**	**18 months**
Control	201.2 ± 9.9	104.8 ± 10.6	98.5 ± 11.8
Ipratropium	147.5 ± 17.5	83.6 ± 11.4	93.3 ± 7.4
Acetylcholine	276.7 ± 25.6	114.0 ± 2.5	107.9 ± 10.0
Ip + ACh	237.4 ± 9.3	101.8 ± 6.8	102.3 ± 9.3

Table 6.2: Time in seconds for 3, 12 and 18 month rat ventricular myocytes to undergo depolarisation following sustained, laser induced, oxidative stress and ipratropium (1 x 10^{-7} M) ± acetylcholine (1 x 10^{-7} M) administration. n=6 animals for all groups, from which between 10-15 myocytes were analysed.



B

Time taken to depolarisation (secs)

Group

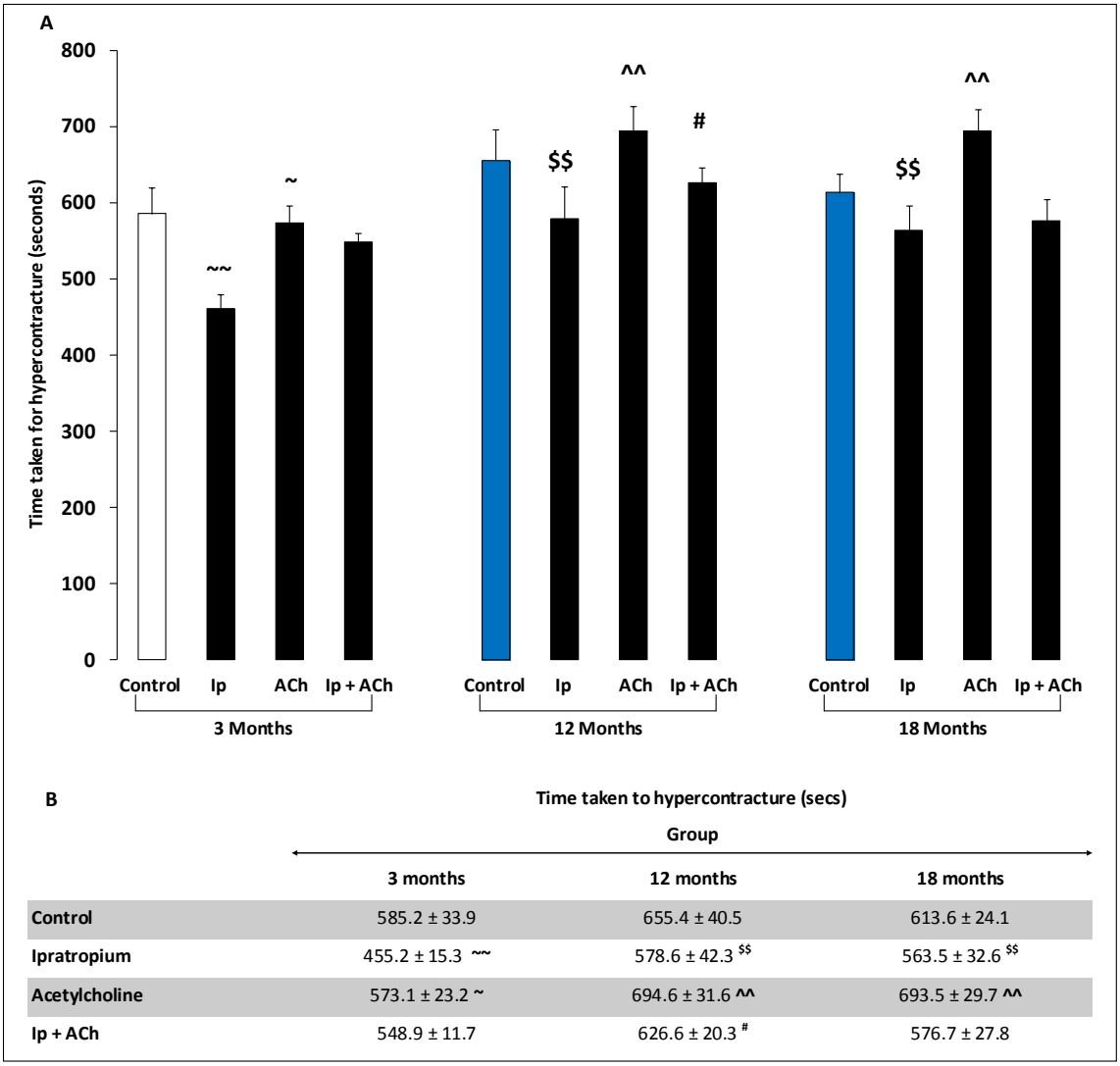
	3 months	12 months	18 months
Control	201.2 ± 9.9	104.8 ± 10.6 **	98.5 ± 11.8 **
Ipratropium	147.5 ± 17.5 ~	83.6 ± 11.4 $$	93.3 ± 7.4 $$
Acetylcholine	276.7 ± 25.6 ~	114.0 ± 2.5 ^^	107.9 ± 10.0 ^^
Ip + ACh	237.4 ± 9.3 ~~	101.8 ± 6.8 ##	102.3 ± 9.3 ##

Figure 6.7: Myocyte model of oxidative stress model conducted in 3, 12 and 18 month old Sprague Dawley rat ventricular myocytes. **A.** Effect of ipratropium (Ip, 1 x 10^{-7} M) ± ACh, (1 x 10^{-7} M) on time to depolarisation and **B.** Values for time to depolarisation under the conditions of sustained oxidative stress. ~ $p<0.05$, ** and ~~ $p<0.01$ vs. 3 month control, $$ $p<0.01$ vs. 3 month Ip, ^^ $p<0.01$ vs. 3 month ACh, ## $p<0.01$ vs. 3 month Ip+ACh. Values expressed as mean + SEM, n=6 animals, with between 10 and 15 myocytes measured, per animal, for each group.

As previously shown (Chapter 4), the time to hypercontracture, following ipratropium treatment, was significantly less than the control in the 3 month group. The data for hypcontracture is presented in Figure 6.8 (**B**) and Table 6.3. Interestingly, the time to hypercontracture in both the 12 and 18 month old group controls were statistically indistinct from the 3 month old control (655.4 ± 40.5 s and 613.6 ± 24.1 s, 12 and 18 months respectively vs. 585.3 ± 33.9 s 3 months). Also, within the 12 and 18 month age groups, there was no significance between the groups treated with ipratropium ± acetylcholine in comparison with the respective control group. However, both the aged (12 and 18 month) ipratropium and acetylcholine treatment groups showed a significant increase in time to hypercontracture in comparison with the same treatment groups from the 3 month old study. In the 12 month old group, there was a significance increase in the time taken to hypercontracture in the group concomitantly treated with ipratropium and acetylcholine in comparison with the equivalent 3 month old treatment group. However, the 18 month old rats, of the same treatment group, were statistically indistinct from both the 3 and 12 month old groups.

	Time taken to hypercontracture (secs)		
	3 months	**12 months**	**18 months**
Control	585.2 ± 33.9	655.4 ± 40.5	613.6 ± 24.1
Ipratropium	455.2 ± 15.3	578.6 ± 42.3	563.5 ± 32.6
Acetylcholine	573.1 ± 23.2	694.6 ± 31.6	693.5 ± 29.7
Ip + ACh	548.9 ± 11.7	626.6 ± 20.3	576.7 ± 27.8

Table 6.3: Time in seconds for 3, 12 and 18 month rat ventricular myocytes to undergo hypercontracture following sustained, laser induced, oxidative stress and ipratropium (1×10^{-7} M) ± acetylcholine (1×10^{-7} M) administration. n=6 animals for all groups, from which between 10-15 myocytes were analysed.



B

Time taken to hypercontracture (secs)

	Group		
	3 months	12 months	18 months
Control	585.2 ± 33.9	655.4 ± 40.5	613.6 ± 24.1
Ipratropium	455.2 ± 15.3 ~~	578.6 ± 42.3 $$	563.5 ± 32.6 $$
Acetylcholine	573.1 ± 23.2 ~	694.6 ± 31.6 ^^	693.5 ± 29.7 ^^
Ip + ACh	548.9 ± 11.7	626.6 ± 20.3 #	576.7 ± 27.8

Figure 6.8: Myocyte model of oxidative stress model conducted in 3, 12 and 18 month old Sprague Dawley rat ventricular myocytes. **A.** Effect of ipratropium (Ip, 1 x 10^{-7} M) ± ACh, (1 x 10^{-7} M) on time to hypercontracture and **B.** Values for time to hypercontracture under the conditions of sustained oxidative stress. ~ p<0.05 and ~~ p<0.01 vs. 3 month control, $$ p<0.01 vs. 3 month Ip, ^^ p<0.01 vs. 3 month ACh, # p<0.05 vs. 3 month Ip+ACh. Values expressed as mean + SEM, n=6 animals, with between 10 and 15 myocytes measured, per animal, for each group.

6.3.3 The role of mPTP opening in aged rat hearts following ipratropium ± cyclosporin A administration in a model of oxidative stress

It was previously demonstrated in Chapter 5 that CsA is capable of partially reversing the observed ipratropium induced myocardial injury, in 3 month adult Sprague Dawley rats. CsA is well documented as a cardioprotective agent and links closure of the mPTP to preservation of myocyte viability under conditions of oxidative stress, such as during myocardial ischaemia/reperfusion (Cohen, Yang and Downey 2008, Crompton 1999, Halestrap 2009, Halestrap and Pasdois 2009, Hausenloy, Boston-Griffiths and Yellon 2011, Hausenloy et al. 2002). However, it has previously been demonstrated that CsA is incapable of eliciting cytoprotective effects in the aged myocardium following such cellular stresses (Liu et al. 2011).

The aim of this study was to ascertain whether the action of CsA was still able to elongate the times to depolarisation and hypercontracture, indicative of preservation of mitochondrial integrity as observed in adult (3 month) rats. Also, to identify whether CsA can limit the observed reduction in times for depolarisation and hypercontracture (as observed in 3 month rats) due to ipratropium administration in aged rat hearts (12 and 18 months).

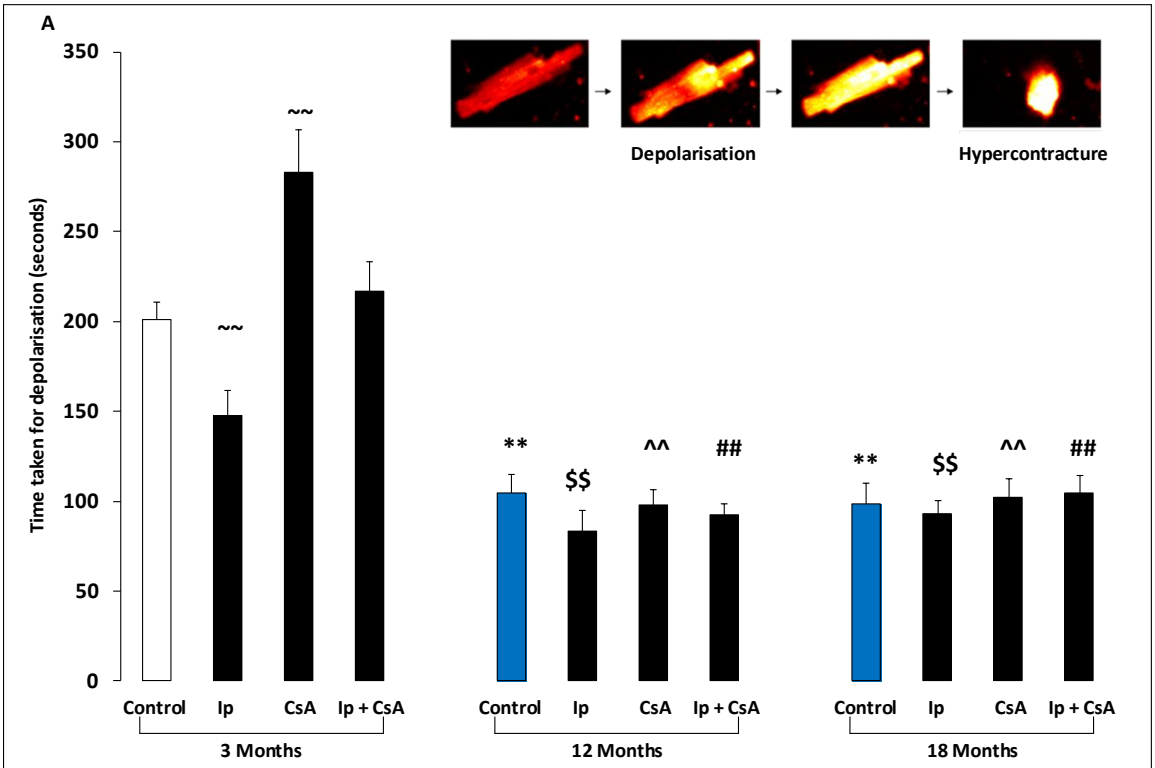
The data from this study is shown in Figure 6.8. Panel **A** represents the time for depolarisation to occur following the onset of laser induced oxidative stress. In comparison with the 3 month control group, the time for depolarisation to occur is significantly decreased in both the 12 and 18 month age groups (104.8 ± 10.6 s and 98.5 ± 11.8 s, 12 and 18 months respectively vs. 201.2 ± 9.9 s 3 months, $p<0.01$), as previously shown in Figure 6.7, **A**. As previously described ipratropium significantly

decreased time to depolarisation in comparison with the untreated control in the 3 month age group, whereas, CsA was capable of significantly prolonging the time for depolarisation (283.1 ± 24.0 s, CsA, $p<0.01$). The co-administration of ipratropium and CsA had an affect which attenuated the reduction in time to depolarisation due to ipratropium administration such that it was statistically indistinct from the control group (Figure 6.9).

In the 12 and 18 month groups, there was a significant decrease in time to depolarisation ($p<0.01$ for all groups) in comparison with the respective 3 month treatment groups. However, there was no significant change in comparison with the control of the same age group observed with any treatment group. There was also no significant change between treatment groups within respective age groups (Table 6.4).

	Time taken to depolarisation (secs)		
	3 months	**12 months**	**18 months**
Control	201.2 ± 9.9	104.8 ± 10.6	98.5 ± 11.8
Ipratropium	147.5 ± 17.5	83.6 ± 11.4	93.3 ± 7.4
Cyclosporin A	283.1 ± 24.0	98.2 ± 8.8	102.1 ± 10.9
Ip + CsA	217.0 ± 16.4	92.7 ± 6.1	104.5 ± 9.8

Table 6.4: Time in seconds for 3, 12 and 18 month rat ventricular myocytes to undergo depolarisation following sustained, laser induced, oxidative stress and ipratropium (1×10^{-7} M) ± CsA (2×10^{-7} M) administration. n=6 animals for all groups, from which between 10-15 myocytes were analysed.



B

Time taken to depolarisation (secs)

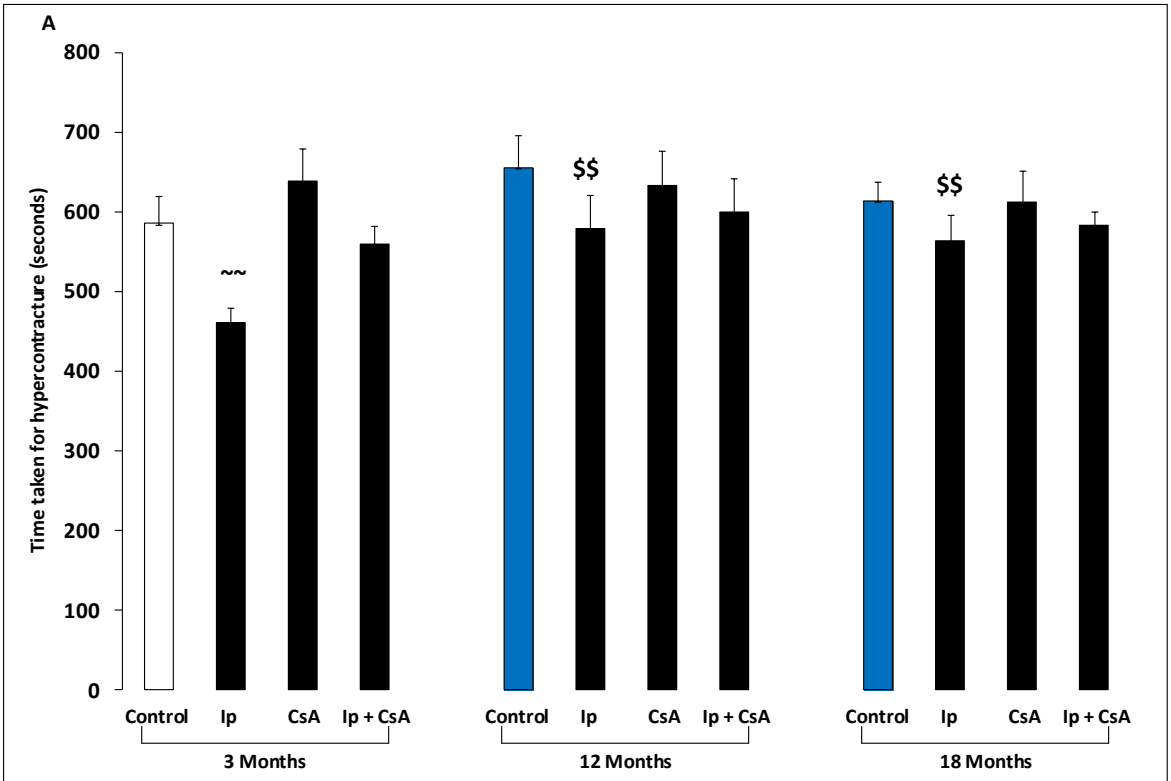
	Group		
	3 months	**12 months**	**18 months**
Control	201.2 ± 9.9	104.8 ± 10.6 **	98.5 ± 11.8 **
Ipratropium	147.5 ± 17.5~	83.6 ± 11.4 $$	93.3 ± 7.4 $$
Cyclosporin A	283.1 ± 24.0 ~	98.2 ± 8.8 ^^	102.1 ± 10.9 ^^
Ip + CsA	217.0 ± 16.4 ~~	92.7 ± 6.1 ##	104.5 ± 9.8 ##

Figure 6.9: Myocyte model of oxidative stress model conducted in 3, 12 and 18 month old Sprague Dawley rat ventricular myocytes. **A**. Effect of ipratropium (Ip, 1 x 10^{-7} M) ± CsA, (2 x 10^{-7} M) on time to depolarisation and **B**. Values for time to depolarisation under the conditions of sustained oxidative stress. **and ~~ p<0.01 vs. 3 month control , $$ p<0.01 vs. 3 month Ip, ^^ p<0.01 vs. 3 month CsA, ## p<0.01 vs. 3 month Ip+CsA. Values expressed as mean + SEM, n=6 animals, with between 10 and 15 myocytes measured, per animal, for each group.

Panel **B** of Figure 6.10 shows the times taken for hypercontracture to occur in 3, 12 and 18 month Sprague Dawley ventricular myocytes. Following ipratropium treatment, hypercontracture was significantly reduced in 3 month old rats, in comparison with the untreated control group ($p < 0.01$). This was increased (although not significantly) following CsA treatment, in comparison with the control and, although CsA appeared to abrogate the observed ipratropium induced reduction in time, when co-administered, this was also not significant. Unexpectedly, the time taken for hypercontracture was statistically indistinct from the 3 month control in all treatment groups in the 12 and 18 month old myocytes. Also, within the 12 and 18 month age groups there was no significant difference between any treatment group. There was, however, a significant increase in the time taken for hypercontracture to occur in the 12 and 18 month groups treated with ipratropium in comparison with the 3 month ipratropium group ($p < 0.01$) (Figure 6.10, Table 6.5).

	Time taken to hypercontracture (secs)		
	3 months	**12 months**	**18 months**
Control	585.2 ± 33.9	655.4 ± 40.5	613.6 ± 24.1
Ipratropium	455.2 ± 15.3	578.6 ± 42.3	563.5 ± 32.6
Cyclosporin A	638.9 ± 41.0	633.7 ± 43.2	611.8 ± 40.6
Ip + CsA	599.0 ± 23.8	600.0 ± 41.9	583.0 ± 17.4

Table 6.5: Time in seconds for 3, 12 and 18 month rat ventricular myocytes to undergo hypercontracture following sustained, laser induced, oxidative stress and ipratropium (1×10^{-7} M) ± CsA (2×10^{-7} M) administration. n=6 animals for all groups, from which between 10-15 myocytes were analysed.



B

	Time taken to hypercontracture (secs) Group		
	3 months	**12 months**	**18 months**
Control	585.2 ± 33.9	655.4 ± 40.5	613.6 ± 24.1
Ipratropium	455.2. ± 15.3 ~~	578.6 ± 42.3 $$	563.5 ± 32.6 $$
Cyclosporin A	638.9 ± 41.0	633.7 ± 43.2	611.8 ± 40.6
Ip + CsA	599.0 ± 23.8	600.0 ± 41.9	583.0 ± 17.4

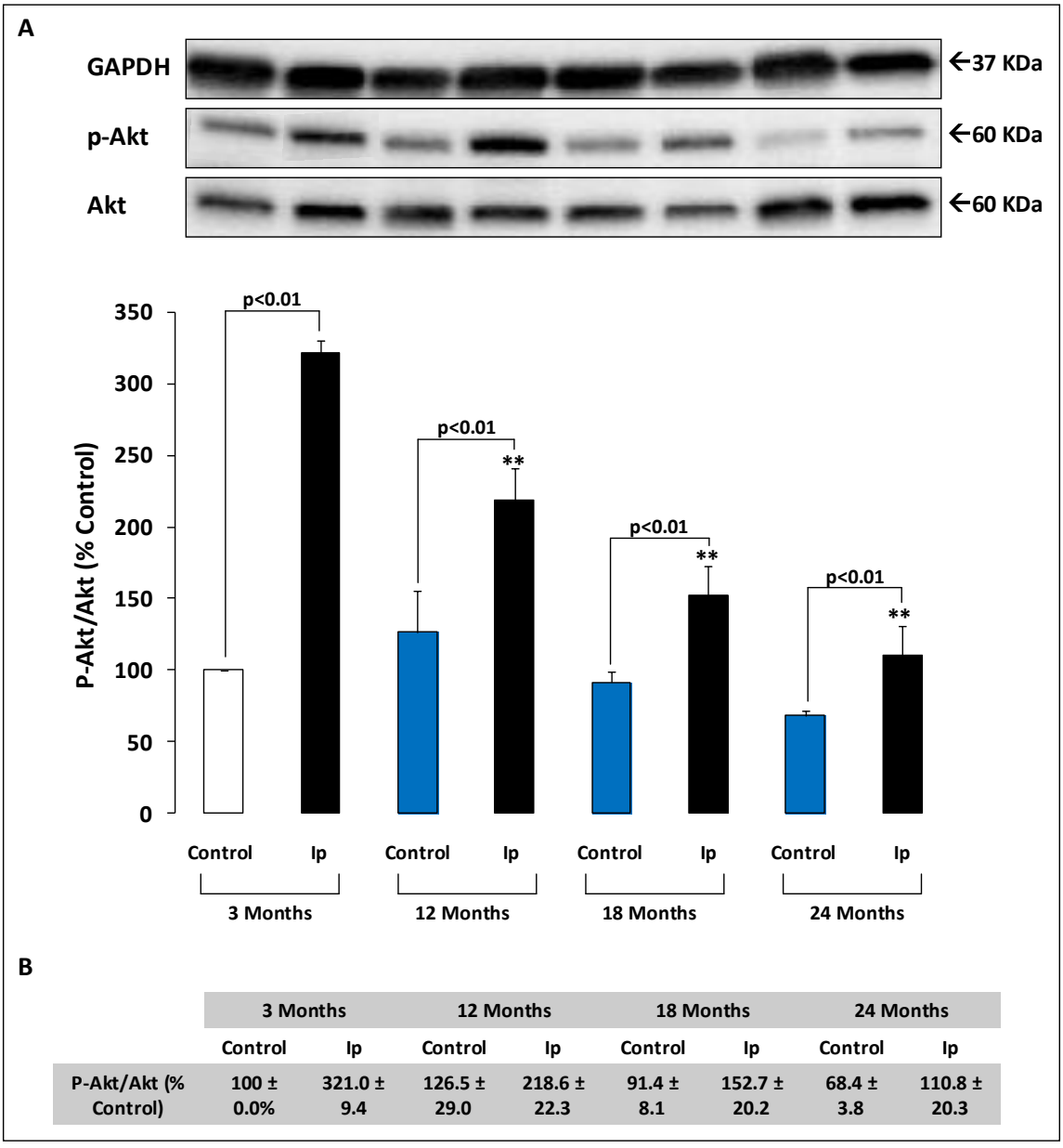
Figure 6.10: Myocyte model of oxidative stress model conducted in 3, 12 and 18 month old Sprague Dawley rat ventricular myocytes. **A**. Effect of ipratropium (Ip, 1 x 10^{-7} M) ± CsA, (2 x 10^{-7} M) on time to hypercontracture and **B**. Values for time to hypercontracture under the conditions of sustained oxidative stress. **and ~~ p<0.01 vs. 3 month control , $$ p<0.01 vs. 3 month Ip, ^^ p<0.01 vs. 3 month CsA, ## p<0.01 vs. 3 month Ip+CsA. Values expressed as mean + SEM, n=6 animals, with between 10 and 15 myocytes measured, per animal, for each group.

6.3.4 Ipratropium bromide induced myocardial injury in aged rat heart is associated with downregulation of phospho-Akt

It was previously reported in Chapter 4, that increased expression of phospho-Akt following ipratropium (1 x 10^{-7} M) administration were associated with ipratropium induced myocardial injury, following 5, 15 and 120 minutes of reperfusion. Due to the identification that ipratropium administration appears to further exacerbate myocardial I/R injury in aged rats (12, 18 and 24 months), the present study aimed to identify whether this exacerbation of injury was associated with changes in Akt levels. Hearts were harvested 15 minutes after the onset of reperfusion where ipratropium (1 x 10^{-7} M) was administered as reperfusion was initiated. Western blot analysis was used to determine the expression levels of phospho-Akt as a percentage of total Akt in the homogenised heart samples.

As shown in Figure 6.11, **A**, in all four age groups, hearts subjected to 35 minutes ischaemia and ipratropium (1 x 10^{-7} M) administration at the onset of reperfusion showed a statistically significant increase in p-Akt in comparison with control I/R hearts for the corresponding age group (Fig. 6.11, **B**, $p<0.01$ for all groups).

Interestingly, there was no significant difference in phospho-Akt levels in comparison with the 3 month I/R control in the I/R control groups in any of the other age groups. However, in contrast to this, there was what appeared to be an age-dependent reduction in phospho-Akt levels compared with the 3 month group in the groups exposed to I/R protocol and ipratropium (1 x 10^{-7} M) administration (321.0 ± 9.4% (3 months) vs. 218.6 ± 22.3% (12 months), 152.7 ± 20.2% (18 months) and 110.8 ± 20.3% (24 months), $p<0.01$).



B

	3 Months		12 Months		18 Months		24 Months	
	Control	Ip	Control	Ip	Control	Ip	Control	Ip
P-Akt/Akt (% Control)	100 ± 0.0%	321.0 ± 9.4	126.5 ± 29.0	218.6 ± 22.3	91.4 ± 8.1	152.7 ± 20.2	68.4 ± 3.8	110.8 ± 20.3

Figure 6.11: The effect of ipratropium (1 x 10^{-7} M) treatment on the levels of phosphorylated Akt following 35 minutes regional ischaemia and 15 minutes reperfusion in hearts taken from 3, 12, 18 and 24 month old male Sprague Dawley rats. **A**. Shows corresponding representative blots for GAPDH, p-Akt and total Akt for all groups and graphical representation of mean + SEM of p-Akt/Akt as a percentage of the 3 month control. **B**. Numerical values for all groups. ** p<0.01 vs. 3 month ipratropium treated group. Statistical difference between ipratropium treated groups and respective control depicted on graph. n=3 for all groups.

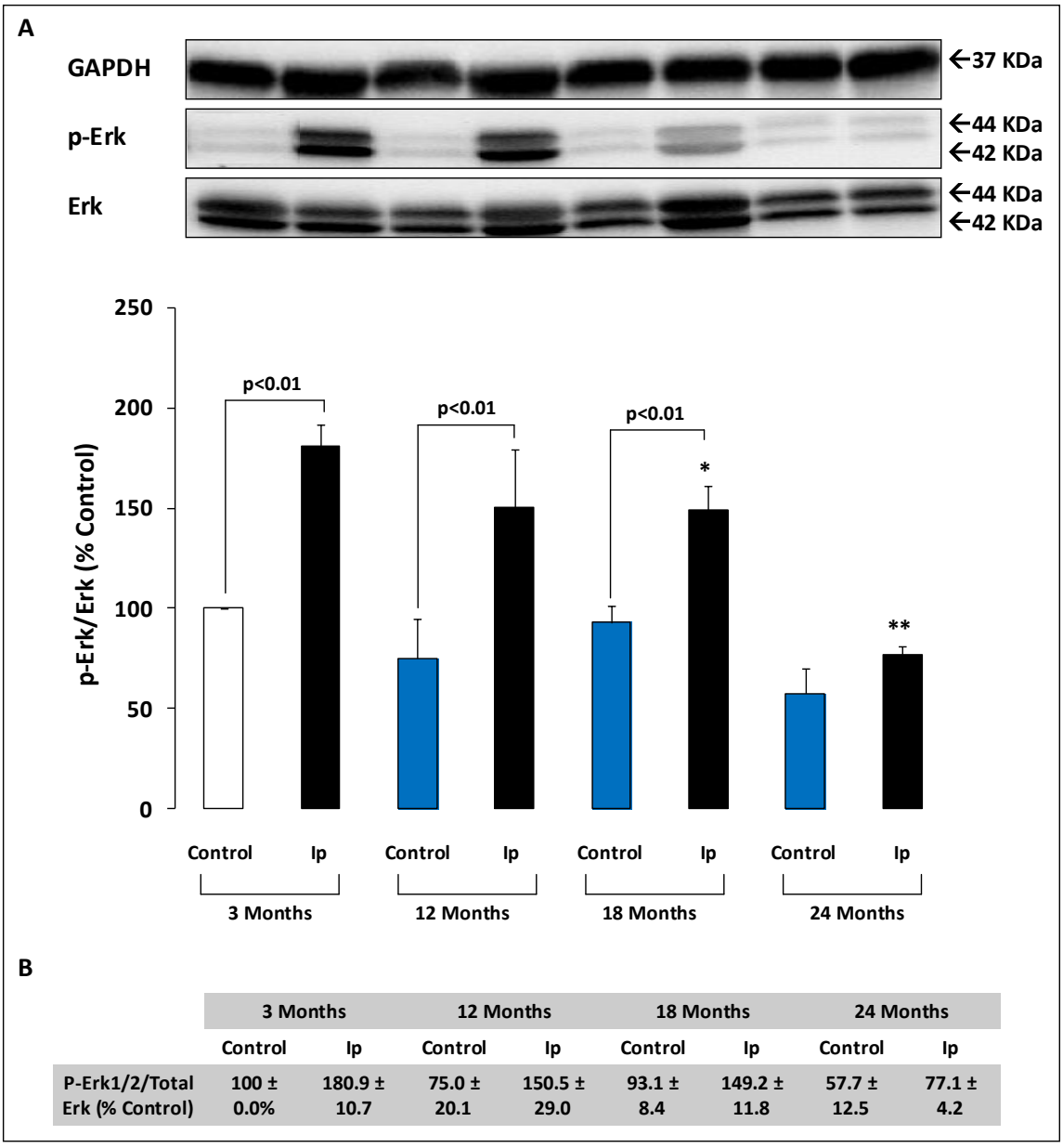
6.3.5 Ipratropium bromide induced myocardial injury in aged rat heart is associated with downregulation of phospho-Erk 1/2

In chapter 4, following 5, 15 and 120 minutes reperfusion, ipratropium administration was associated with a statistical increase in levels of phospho-Erk1/2. In this study, 3, 12, 18 and 24 month old Sprague Dawley rat hearts were subjected to 35 minutes regional ischaemia and 15 minutes reperfusion, in the absence and presence of ipratropium (1 $x10^{-7}$ M) at reperfusion, and harvested for Western blot analysis (as previously described). These studies were conducted in order to elucidate whether there was involvement of Erk 1/2 in the observed exacerbation of ipratropium induced myocardial injury in the aged myocardium.

There was no observed statistical difference in levels of phosphorylation of Erk between the 3, 12 or 18 month control groups (values in Figure 6.12 **B**). However, in the 24 month group, levels of phospho Erk were significantly less than in the 3 month control group (100.0 ± 0.0%, 3 months vs 57.7 ± 12.5%, 24 months, $p<0.01$).

As previously observed, the administration of ipratropium significantly increased Erk phosphorylation in comparison with the untreated control in the 3 month group (100.0 ± 0.0%, control vs. 180.9 ± 10.7%, Ip, $p<0.01$). In both the 12 and 18 month groups, the administration of ipratropium significantly increased the level of phospho-Erk in comparison with the respective untreated control of the same age group (values in figure 6.12 **B**, both $p<0.01$). In the 24 month age group, despite a small increase in Erk phosphorylation in comparison with the control, the difference between the two groups was not significant.

Interestingly, the levels of Erk phosphorylation in the ipratropium treated groups appeared to decline in an age dependent manner. In particular, in the 18 and 24 month groups, the levels of phospho-Erk were significantly less than in the 3 month ipratropium treatment group (values in Figure 6.12, $p<0.05$, 18 months, $p<0.01$, 24 months).



B

	3 Months		12 Months		18 Months		24 Months	
	Control	Ip	Control	Ip	Control	Ip	Control	Ip
P-Erk1/2/Total Erk (% Control)	100 ± 0.0%	180.9 ± 10.7	75.0 ± 20.1	150.5 ± 29.0	93.1 ± 8.4	149.2 ± 11.8	57.7 ± 12.5	77.1 ± 4.2

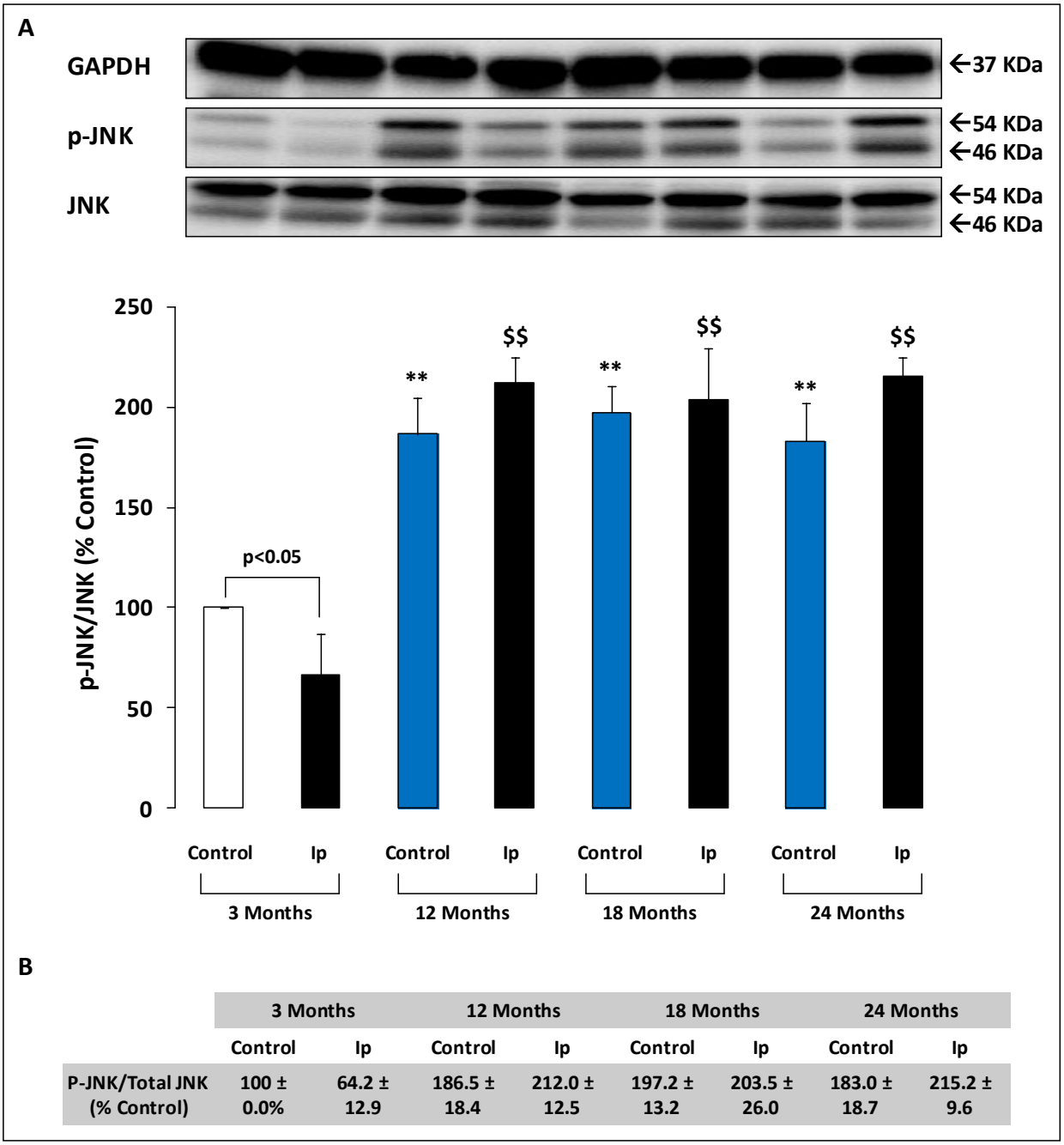
Figure 6.12: The effect of ipratropium (1 x 10^{-7} M) treatment on the levels of phosphorylated Erk1/2 following 35 minutes regional ischaemia and 15 minutes reperfusion in hearts taken from 3, 12, 18 and 24 month old male Sprague Dawley rats. **A.** Shows corresponding representative blots for GAPDH, p-Erk1/2 and total Erk1/2 for all groups and graphical representation of mean + SEM of p-Erk1/2/Erk1/2 as a percentage of the 3 month control. **B.** Numerical values for all groups. * p<0.05 and ** p<0.01 vs. 3 month ipratropium treated group. Statistical difference between ipratropium treated groups and respective control depicted on graph. n=3 for all groups.

6.3.6 Ipratropium bromide induced myocardial injury in aged rat heart is associated with an increase in phosphorylation of JNK

In chapter 4, following 5, 15 and 120 minutes reperfusion, ipratropium administration was associated with a statistical decrease in levels of phospho-JNK, following 15 minutes reperfusion. In this study, 3, 12, 18 and 24 month old Sprague Dawley rat hearts were subjected to 35 minutes regional ischaemia and 15 minutes reperfusion, in the absence and presence of ipratropium (1 $x10^{-7}$ M) at reperfusion, and harvested for Western blot analysis (as previously described). These studies were conducted in order to elucidate whether there was involvement of JNK in the observed exacerbation of ipratropium induced myocardial injury in the aged myocardium.

In the 12, 18 and 24 month untreated control groups, there were significantly higher levels of phospho-JNK in comparison with the 3 month control group ($p<0.01$ vs. 3 month control for all groups, values in Figure 6.13 B). In the ipratropium treatment groups, there was also a significant increase in comparison with the 3 month group with all of the other ipratropium treatment groups *$p<0.01$ vs. 3 month ipratropium group for all groups, values in Figure 6.13 B).

Interestingly, with the exception of the 3 month group, there was no statistical difference between the control and ipratropium groups in the 12, 18 or 24 month rats.



B

	3 Months		12 Months		18 Months		24 Months	
	Control	Ip	Control	Ip	Control	Ip	Control	Ip
P-JNK/Total JNK (% Control)	100 ± 0.0%	64.2 ± 12.9	186.5 ± 18.4	212.0 ± 12.5	197.2 ± 13.2	203.5 ± 26.0	183.0 ± 18.7	215.2 ± 9.6

Figure 6.13: The effect of ipratropium (1 x 10^{-7} M) treatment on the levels of phosphorylated JNK following 35 minutes regional ischaemia and 15 minutes reperfusion in hearts taken from 3, 12, 18 and 24 month old male Sprague Dawley rats. A. Shows corresponding representative blots for GAPDH, p-JNK and total JNK for all groups and graphical representation of mean + SEM of p-JNK/JNK as a percentage of the 3 month control. B. Numerical values for all groups. ** p<0.01 vs. 3 month control group, $$ p<0.01 vs. 3 month ipratropium treatment group. Statistical difference between respective control depicted on graph. n=3 for all groups.

6.4 Chapter Discussion

Globally, by the year 2050, the number of individuals who fall into the "elderly" population (aged 60 or above) is projected to be 1.5 billion. As previously described, aging not only represents an increase in the risk of myocardial syndromes, but also enhances susceptibility to adverse clinical outcomes. In the context of myocardial I/R, aging has been shown as a critical determinant in the exacerbation of I/R injury (Lesnefsky et al. 1994, Lucas and Szweda 1998). Within the heart, changes in cellular metabolism in senescent myocytes leads to alterations of myocardial metabolism, mitochondrial function (Jian et al. 2011), increases in ROS and deficiencies in Ca^{2+} signalling. The contribution of these changes in the context of I/R leads to deregulation of downstream cellular survival pathways (Korzick and Lancaster 2013, Tani et al. 1999).

In addition to this, COPD is, on average, diagnosed at 67 years of age. However, despite the frequent co-morbidity of COPD with concurrent IHD and the knowledge that MI accounts for a major cause of death in COPD patients, as well as the previous mentioned alterations in senescent myocytes, there are not currently different therapies for elderly COPD patients. It has also been observed that, in older patients, left ventricular function is compromised, which represents a further risk factor for the development of IHD (Gardin and Lauer 2004). The aim of the present study was to identify the effects of ipratropium on myocardial injury following I/R, in the hearts of aged (12, 18 and 24 month) rats.

The major finding of the work presented in this chapter is that, within the aged rat groups (12, 18 and 24 months), myocardial injury following simulated I/R is

significantly exacerbated in comparison with the 3 month, adult rat, groups. This was observed as a significant increase in I/R% in the Langendorff isolated perfused heart model (12, 18 and 24 months vs. 3 month group), as well as a significant decrease in time to depolarisation, indicative of loss of mitochondrial integrity, in the cardiac myocyte model of oxidative stress (12 and 18 months vs. 3 month group). These observations support a growing body of research which shows that aging itself exacerbates I/R injury (Poulose and Raju 2014). Coupled with this, it has also been demonstrated that protection afforded by ischaemic pre-conditioning is lost in the aged myocardium (van den Munckhof et al. 2013). Under naïve conditions, there is a reduction in myocyte mitochondrial activity, as well as a reduction in the number of mitochondria. This implies there is a reduced oxidative phosphorylation (attributed to decreased complex III and IV activity), therefore contributing to a baseline increase in oxidative stress, even in the absence of myocardial injury (Boengler, Schulz and Heusch 2009).

In contribution to this, Ca^{2+} handling is deregulated in the aged myocardium, due to age associated increases in NCX activity as well as a reduction in SERCA expression (Janczewski, Spurgeon and Lakatta 2002). This promotes endogenous Ca^{2+} overloading in the absence of disease which, following I/R, is further exacerbated.

Primarily, the mechanism of mPTP opening has been shown as a determinant for myocyte fate at the onset of reperfusion, following an ischaemic insult (Halestrap 2009). In conjunction with increases in intracellular Ca^{2+} and ROS generation, the reduction in mitochondrial membrane integrity following mPTP opening promotes myocyte loss by both apoptosis and necrosis (Halestrap 2009). ROS induced injury

following I/R is thoroughly documented, as is the contribution of increases in Ca^{2+} (Chen et al. 2006, Chen et al. 2011). Within the senescent myocardium, the underlying elevated ROS and Ca^{2+} levels, are further exacerbated during I/R, which therefore promotes mPTP opening at reperfusion to an extent which is greater than that in the young myocardium. This therefore leads to further infarct development due to higher levels of apoptosis and necrosis, as observed in the Langendorff studies. The myocyte model of oxidative stress used in this work also supports the literature as there was a significant reduction in time to depolarisation following sustained oxidative stress, implying that the mitochondria are less resilient in comparison with those in the 3 month group. This has been attributed to underlying increases in ROS and Ca^{2+} in the aged cardiac myocytes.

Interestingly, there was no aged-related significant difference in hypercontracture observed in comparison with the 3 month group. In chapters 4 and 5, it was observed that, despite large differences following different pharmacological treatments in the time to depolarisation, the variation in time to hypercontracture between groups was much less. It is postulated that this is due to the time taken for elevated Ca^{2+} levels to reduce following I/R or oxidative stress. This is dependent on the restoration of NCX and IP3R function which requires reactivation of the Na^{+}-K^{+}-pumps by ATP, thereby slowly restoring the Na^{+} gradient prior to the NCX being able to extrude the excess cytosolic Ca^{2+} (Zaugg, Schaub and Foex 2004, Murphy and Steenbergen 2008). However, irrespective of the severity of ischaemic injury, the time frame for this does not particularly vary. A possible explanation for the results presented here is that, within the aged myocardium, although resting levels of Ca^{2+} are significantly higher

than in the young heart, the time frame for the restoration of baseline Ca^{2+} levels, following re-establishment of NCX and IP3R function, remains indifferent from that in the adult (3 month) group.

In the Langendorff model, there was a further increase in I/R% in comparison with the respective untreated control in the aged myocardium (significant in the 18 and 24 month groups). This is in accordance with the data from the 3 month group whereby ipratropium was observed to exacerbate myocardial injury following I/R (Chapter 3). Myocardial injury in the aged myocardium was already observed to be statistically exacerbated, as described above. In the 3 month group, the exacerbation of I/R% following ipratropium administration was attributed to blockage of endogenous ACh signalling, therefore preventing activation of pro-survival kinases such as (PI3K)-Akt and Erk1/2 (components of the RISK pathway). In the case of the aged study, it is understood that muscarinic receptors within the myocardium decrease with age (Brodde et al. 1998, Lo et al. 2001). It is therefore possible that the competitive action of ipratropium at the muscarinic receptors in the aged myocardium is, therefore, sufficient to further diminish downstream, protective signalling cascades from endogenous ACh, thereby contributing to a further exacerbation of myocardial injury in this context.

It is plausible that the exacerbation of infarct development following ipratropium injury in the aged myocardium is also due to disruption of RISK signalling, which, once activated, is linked to closure of the mPTP, therefore maintaining mitochondrial function and salvage of cardiac myocytes. However, in the aged myocardium, protection via mechanisms which prevent mPTP opening appear to be lost (Liu et al.

2011b). This is primarily due to the increased susceptibility for mPTP opening due to elevated levels of ROS and Ca^{2+} (Liu et al. 2011b).

Further to this, it has also been demonstrated that mPTP opening promotes myocyte loss, on the whole, by primary necrosis with apoptotic death only slightly contributing (Kung, Konstantinidis and Kitsis 2011). However, within the aged myocardium, it has been demonstrated that, following an ischaemic insult, the contribution of necrosis to myocardial injury is indistinct from that observed in younger rats, however, the increase in apoptosis is greatly increased (Kajstura et al. 1996). This has been accompanied by age associated increases in essential components of the intrinsic apoptotic cascade, such as Bcl-2, Bax, caspase-3 and cytochrome c (Liu et al. 2002, Phaneuf and Leeuwenburgh 2002, Liu et al. 2011b) at both the cellular and molecular level as mRNA levels of Bax are disproportionately increased in comparison with Bcl-2 (Liu et al. 2002).

The role for the mPTP in ipratropium induced myocardial injury in the aged heart was studied via the model of oxidative stress (Figures 6.8 – 10). As already described, there was a significant reduction in time to depolarisation in the control aged groups in comparison with the respective 3 month group. In comparison with the respective treatment groups, in both the 12 and 18 month groups, all drug treatment groups (Ip, ACh, CsA and Ip ± ACh or CsA) showed a significant ($p<0.01$ for all groups) decrease in the time taken for depolarisation to occur in comparison with the respective, 3 month, treatment group. However, within the 12 and 18 month groups, there was no significant change in depolarisation observed between any of the treatment groups within the same age group.

As previously described, following aging, ROS and Ca^{2+} levels are increased, leading to an increased susceptibility for the mPTP to open at the onset of reperfusion. In the study where ipratropium and ACh were investigated, it is possible that the inability for ACh to protect is due to the down-regulation of cardiac mAChR receptors. This may mean that activation of components of the RISK signalling cascade are not as potently activated following muscarinic stimulation as in young (3 month) rats. Due to the intrinsic link that RISK pathway activation prevents mPTP opening, this data implies that this limitation of pro-survival muscarinic signalling may be sufficient to prevent preservation of mitochondrial membrane integrity through the delay of mPTP opening. Further to this, opening of K_{ATP} channels have been shown to play a role in acetylcholine mediated pre-conditioning due to inhibition of mPTP opening (Qian et al. 1996, Hausenloy et al. 2002). However, in the aged myocardium, a reduction in K_{ATP} channels has been shown (Ranki et al. 2002) and also, isoflurane (an anaesthetic known to induce cardio-protection via K_{ATP}) is significantly less effective following aging (Mio et al. 2008). It is therefore possible that the impaired function of K_{ATP} channels, due to aging, is able to "over-ride" the protective properties of ACh, thereby abolishing the protective effect of ACh on the mPTP.

In the 3 month group, ipratropium accelerates mPTP opening. However, in this study, further contribution to the decrease in time to depolarisation following ipratropium administration does not occur. As with ACh, it could be that the age-associated reduction of mAChRs in the aged myocardium means that ipratropium is unable to exert further injury via disruption of the mitochondria through muscarinic signalling.

In the context of the CsA studies, it was also observed that CsA was unable to protect against the prevention of mPTP opening in the aged (12 and 18 month) groups, whereas it was observed in chapter 5 that CsA could potently protect against mPTP opening as well as ipratropium mediated myocardial injury.

Cyclosporin A (CsA) inhibits mPTP opening via binding to cyclophilin-D, which therefore elicits CsA mediated cardioprotection (Xie and Yu 2007, Halestrap and Richardson 2014). This has been widely documented and observed, as significant decreases in infarct size as well as modulation of critical mediators of I/R injury, following CsA administration. In the context of the aged myocardium, CsA is incapable of reducing infarct size. In conjunction with this, NAD^+ loss following mPTP opening was not different in the aged hearts following I/R with or without CsA administration (Liu et al. 2011b). Given that the cardioprotective effects of CsA can be inhibited by elevations of Ca^{2+}, it is possible that the inability for CsA to protect in the aged myocardium involves a similar mechanism as Ca^{2+} levels are significantly higher following I/R in aged hearts in comparison with young hearts.

It is proposed that CsA protects against mPTP opening via a mechanism which inhibits cyclophilin D from associating with the ANT (Halestrap 2009), as such, it is possible that the observed defects within the mitochondria and increases in oxidative damage due to aging are sufficient that the mitochondria are less resilient to oxidative stress, such that the interaction of CsA with cyclophilin D is unable to protect (Liu et al. 2011b).

There is a multitude of evidence to suggest a down-regulation of GPCR expression as a consequence of aging. From an intracellular signalling perspective, the limitation of cytoprotective, GPCR mediated, signalling is either due to a reduction in receptors of

loss of sensitivity to agonist binding (Peart et al. 2014). The correlation of the expression of mAChRs with age is of primary interest. As already stated, mAChR expression has been shown to decrease with age.

In this study, levels of the pro-survival kinases Akt and Erk1/2, as well as the stress induced kinase JNK, were ascertained in 3, 12, 18 and 24 month rats following 35 minutes regional ischaemia and 15 minutes reperfusion. This is due to the primary involvement of Akt and Erk1/2 in pro-survival signalling cascades following I/R as well as the known association of JNK activation following cellular stresses (including I/R). All three kinases are also known effectors of downstream signalling following muscarinic receptor activation by acetylcholine. Further to this, significant differences in levels of Akt, Erk1/2 and JNK phosphorylation were previously observed in 3 month rats following I/R and ipratropium administration.

The involvement of Akt and Erk signalling in the aged myocardium has been shown as an important determinant in sevoflurane mediated post-conditioning (Li et al, 2013). It has also been demonstrated that, within the murine and human myocardium, there is a repressed tolerance to ischaemia which appears to be due to age-related differences in cyto-protective, kinase signalling (Peart et al. 2014). The data presented here support this as Akt and Erk1/2 are both activated at lower levels (even in the absence of ipratropium) following I/R in the aged tissues compared with the three month controls. It is also interesting that JNK, a stress induced kinase, appears to have a role in the myocardial injury recorded in this study. However, previously (Chapters 4 and 5) it appearsed as though JNK was not instrumental in mediating ipratropium induced myocardial injury. It is therefore likely thar JNK is activated at higher levels within the

aged myocardium, as previously described (Xu et al. 2014), which contributes to myocardial injury, even in the absence of a cardio-toxic agent, such as ipratropium.

Chapter 7 General Discussion

7.1 Summary of Findings

Ipratropium bromide has been administered as a treatment for COPD and emergency adjunct therapy for asthma since 1987 (Restrepo 2007). The primary aim of this work was to identify whether there was a cellular basis for the observed increased risk of adverse cardiovascular outcomes in the setting of underlying heart conditions, following ipratropium administration, as outlined in the meta-analysis conducted by Singh and colleagues (Singh et al. 2008). Specifically, this was investigated in the context of ischaemia/reperfusion injury in order to ascertain whether, in a non-clinical *ex vivo* heart model, injury following regional ischaemia and reperfusion was exacerbated due to ipratropium administration.

The rationale behind the use of this model was that many patients who receive ipratropium may also be at risk of myocardial infarction if they have underlying IHD (Huiart, Ernst and Suissa 2005, Maclay, McAllister and Macnee 2007, Macnee, Maclay and McAllister 2008). The co-morbidity of IHD with COPD remains the biggest risk factor for death by myocardial infarction (MI) in COPD patients (Zielinski et al. 1997). There is an increasing wealth of clinical studies which indicate that anti-muscarinic treatments (such as ipratropium) are responsible for an increased risk of both occurrence and severity of adverse cardiovascular events (Singh et al. 2008, Singh et al. 2013). As such, this work aimed to identify whether this was due to ipratropium interacting with cellular signalling pathways involved in the clinical manifestation of MI. In a non-clinical, although clinically relevant, study, this was addressed by using *in vitro* rat myocardial models of simulated ischaemia and reperfusion.

From this respect, this work has identified that under normoxic conditions ipratropium does not appear to influence the cellular function of cardiac myocytes, with the exception that at 1 x 10^{-6} M and 1 x 10^{-7} M ipratropium, following administration for 60 minutes, LVDP showed significant increases in comparison with the untreated controls and heart rate was significantly increased (also following 60 minutes exposure to ipratropium) in the group treated with 1 x 10^{-6} M ipratropium. This is in accordance with previous work which has shown ipratropium to possess positive inotropic and chronotropic effects (Windom 1990). This is potentially due to an impairment of the NCX and, therefore, mediated via Ca^{2+} influx, as previously shown in the context of cardiotoxicity where physostigmine (an AChE inhibitor) potentiated negative inotropic and chronotropic responses within rat atria, but this was antagonised with the addition of atropine (Mazumder et al. 1997).

Interestingly, in the studies where hearts were subjected to the I/R protocol, ipratropium administration did not appear to have any haemodynamic effect, although there was observed exacerbation of infarct development in comparison with the untreated control hearts subjected to the same protocol. It is speculated here that the absence of haemodynamic effects in these studies is as a result of overall cardiac dysfunction following ipratropium induced injury and, therefore, haemodynamic effects could not be observed in this study.

The release of ACh from the vagus nerve, and subsequent binding to MAChRs in the myocardium, stimulates parasympathetic actions leading to GPCR led decreases of cAMP and activation of K_{ACh} channels (Shultz et al. 2008). Therefore, endogenously, ACh reduces the rate of conduction from the sinoatrial node leading to negative

chronotropy (Nathanson 2008). In contrast to this, *in vivo* and clinical use of muscarinic antagonists prevent the action of ACh and attenuate the vagal nerve stimulation, therefore leading to increases in both heart rate and conduction velocity (Singh et al. 2013). In patients with underlying heart conditions, this can promote arrhythmias and tachycardia. From a clinical perspective, pro-arrhythmic and pro-ischaemic effects due to anti-muscarinic treatments have been observed in patients with underlying IHD (Singh et al. 2013). It is possible that the inotropic and chronotropic effects may contribute to contractile dysfunction, which could represent a further risk factor for the development of MI and IHD. Contractile dysfunction is a major hallmark for the development of many cardiovascular disorders, including conditions involving ischaemic events such as MI and IHD. It is considered that abnormal levels of Ca^{2+} influence not only the contraction of the heart, but also can contribute to mitochondrial Ca^{2+} overload, therefore also initiating mitochondrial damage and oxidative stress (Goldhaber 2001). In addition to this, ipratropium has been shown to stimulate eryptosis (programmed cell death in erythrocytes with mechanistic similarities to apoptosis) via increases in Ca^{2+} (Shaik et al. 2012). In combination, it is therefore possible that ipratropium elicits myocardial damage following I/R through a similar mechanism which stimulates apoptosis via increases in Ca^{2+}. As it is known that the protective properties of ACh are partly due to cholinergic signalling which regulates Ca^{2+} levels (Russo et al. 2014), it is plausible that muscarinic antagonists could promote myocardial injury through mechanisms which cause de-regulation of the same cellular machinery, in this case, Ca^{2+} regulation and signalling.

Within this work it was identified, by use of the MTT assay and flow-cytometry, that ipratropium decreases overall myocyte viability, including both apoptosis and necrosis, which were observed following ipratropium administration. This supports the notion that myocyte death after the initiation of reperfusion occurs through concomitant apoptosis and necrosis (Buja and Weerasinghe 2010, Bishopric et al. 2001) and implies that ipratropium exerts its cardio-toxic effect during reperfusion rather than before. The remainder of this work therefore concentrated on cellular processes which occur during reperfusion in order to try to identify a signalling mechanism for ipratropium induced myocardial injury.

7.1.1 Mechanism of protection by Acetylcholine

In order to further characterise the mechanism by which ipratropium elicits myocardial damage following an ischaemic insult, studies were conducted with ACh and CsA as both agents are known to be cardio-protective, as has been demonstrated in rat, mouse and human cell lines. In particular, ACh is the endogenous agonist of muscarinic receptors (Caulfield and Birdsall 1998).

With use of an ACh assay, it was identified that endogenous ACh was present in the experimental models used. This was necessary as an antagonist does not exhibit efficacy following receptor binding and, therefore, without endogenous ACh present, it is likely that the detrimental effects observed due to ipratropium administration were off-target and not due to disruption of muscarinic signalling.

In the context of cardio-protection, ACh has shown to exert protective effects in various animal models, including rat and mouse following an ischaemic insult (Buys et al. 2003, De Sarno et al. 2003, De Sarno et al. 2005, Styles, Zhu and Li 2005). In particular, this is proposed to be via the M_3 muscarinic receptor subtype present in the heart (Budd et al. 2003, Li et al. 2011, Resende and Adhikari 2009).

Following simulated I/R, ACh was shown to statistically abrogate infarction due to I/R alone, therefore supporting previous work (Sun et al. 2013). ACh was also able to significantly increase the time for depolarisation to occur in the model of oxidative stress, thereby also supporting previous investigations which have shown that ACh elicits its cardio-protective effects via mechanisms which involve the RISK pathway linked to the preservation of mitochondrial integrity due to promoting closure of the mPTP (Sun et al. 2010a).

In the presence of ipratropium, ACh was able to significantly attenuate the observed myocardial damage, this implies that ipratropium is acting at the muscarinic receptors and that blockade of endogenous ACh by ipratropium is sufficient to limit downstream signalling which leads to loss of the protective mechanisms which are normally elicited by ACh. These observations also support other work where it has been shown that ACh mediated cyto-protection can be abolished by muscarinic antagonists (Lim et al. 2007).

In this study ipratropium (a non-selective muscarinic antagonist) was used and so it is not possible to identify whether the ipratropium induced injury is due to a specific action on one subtype of muscarinic receptor, or whether it is a general action on all the subtypes in the heart. By population, M_2 receptors are the most densely distributed of all muscarinic receptors in the myocardium, however the role of M_3

receptors in cardio-protection has been more extensively studied in comparison with the other subtypes (Pan et al. 2012, Wang et al. 2012, Zhao et al. 2010). It actually appears as though M_2 receptors play a minor role in protective signalling following I/R and, therefore it is proposed in this work that it is the antagonist action of ipratropium on the M_3 receptors which is responsible for the observed myocardial injury. In order to fully elucidate this, further work with selective antagonists and, where possible, agonists, for specific subtypes of muscarinic receptors would need to be carried out.

7.1.2 Mechanism of protection by CsA

CsA is known to protect from the mitochondria via a mechanism which prevents opening of the mPTP and was therefore used to help clarify whether ipratropium induced injury has mitochondrial involvement. It is now well established that opening of the mPTP within the first few minutes of reperfusion is a pivotal event in the development of infarction and cardiac myocyte loss following an ischaemic event. Throughout prolonged ischaemia, Ca^{2+} levels steadily rise, mitochondrial [P_i] is high and levels of ATP are significantly reduced due to limitation of oxidative phosphorylation. These events condition the mPTP such that the oxidative stress and further Ca^{2+} accumulation, as well as increases in pH following ischaemia induced acidosis, which occur as a consequence of reperfusion, promote pore formation (Halestrap and Richardson 2014). Upon mPTP opening, cytosolic solutes rapidly move into the mitochondria causing swelling and, eventual rupture, of the outer mitochondrial membrane. This then promotes cardiac myocyte death due to both

apoptosis (following release of mitochondrial pro-apoptotic factors) as well as necrosis (due to inhibition of ATP formation).

In addition to this, protective strategies which target the mPTP have therefore become attractive since the elucidation of the detrimental role of mPTP opening at the onset of reperfusion (Cohen, Yang and Downey 2008, Crompton 1999, Halestrap 2009, Halestrap and Pasdois 2009, Hausenloy, Boston-Griffiths and Yellon 2011, Hausenloy et al. 2002). In this work, 2 x 10^{-7} M CsA was used as this concentration has previously been shown to potently protect against oxidative stress induced myocardial damage due to its ability to associate with CypD and, therefore, restricts mPTP opening. Although in the model used in this work opening of the mPTP was not directly measured, the model used allowed depolarisation and hypercontracture to be measured which are indicative of mPTP opening and myocte rigor due to loss of ATP, respectively (Yellon and Hausenloy 2007). Within this work, it has been demonstrated that ipratropium significantly reduced the time to depolarisation, therefore indicating that ipratropium treatment renders myocytes less resilient to oxidative stress and, at least in part, that ipratropium exerts its detrimental effects on the myocardium due to an action on the mPTP. CsA was shown to provide significant protection against the ipratropium induced injury thereby further supporting that ipratropium induced myocardial injury has an element of mitochondrial involvement.

7.1.3 Signalling proteins

Within this work it was ascertained that in the model of I/R used, ipratropium administration during reperfusion significantly increased levels of p-Akt and pErk1/2. Co-administration of ACh with ipratropium statistically abrogated the observed increase in p-Akt observed with ipratropium alone, however did not significantly abrogate p-Erk1/2 levels at all time points. Interestingly, CsA administration caused significant reductions in p-Akt and p-Erk1/2 levels in comparison with the untreated controls and was unable to significantly abrogate the ipratropium induced increases in p-Akt and p-Erk1/2. It is generally considered that increases in Akt and Erk1/2 activity are protective cellular mechanisms against various cellular insults, including I/R injury (Miyamoto, Murphy and Brown 2009). However, constitutive over activation of Akt has been shown as detrimental in various animal models including rat, (Nagoshi et al. 2005, O'Neill and Abel 2005) and also, previous studies have shown doxorubicin induced elevation of p-Akt and p-Erk1/2 in conjunction with cardiovascular injury (Gharanei et al. 2013). This was attributed to an intrinsic survival mechanism whereby myocytes up-regulate these proteins in order to counteract the toxic effects of doxorubicin. There is evidence that, in part, doxorubicin has anti-muscarinic properties (Sasaki et al. 2010). As such, it is postulated here that the increases in Akt and Erk1/2 activation following ipratropium treatment may be attributed to a similar mechanism as that of doxorubicin.

However, although the study which used wortmannin in conjunction with ipratropium showed entire ablation of p-Akt levels, this was not associated with a decrease in infarct size as observed with ipratropium treatment alone. This implies that although

there is a cellular mechanism which increases Akt phosphorylation following ipratropium administration, this is not a determining factor for an increase in ipratropium induced injury and may simply be a consequence of ipratropium administration but not a requisite for injury. In order to further elucidate this, UO126 should be used in conjunction with ipratropium to ascertain whether inhibiting Erk1/2 activation influences the levels of injury observed as Erk1/2 is able to be activated due to cross-talk following activation of Akt in the signalling cascade.

There is a wealth of evidence which suggests that, despite the well reported role of Akt as a protective kinase (Sussman et al. 2011), paradoxically, chronic Akt activation has been shown to fail to reduce infarction following I/R and has been demonstrated to actually increase infarct size in an isolated heart model of I/R in the mouse (Nagoshi et al. 2005, O'Neill and Abel 2005). Exacerbated levels of Akt phosphorylation have also been identified in patients with heart failure (Nagoshi et al. 2005).Interestingly, in this context, although PI3K inhibition is generally shown to abolish the protective effects of Akt, it also appears that inhibition of PI3K is capable of further exacerbating myocardial injury due to chronic Akt activation (Nagoshi et al. 2005). These data imply that PI3K is necessary for the regulation and promotion of cadioprotection due to Akt signalling and, therefore that protective or detrimental cellular mechanism may be dependent on PI3K rather than the levels of phosphorylated Akt (Sussman et al. 2010). This indirectly supports the current study where it appeared from the wortmannin studies that Akt activation is not a requisite for ipratropium induced myocardial injury. In order to more fully clarify these mechanisms, studies designed to specifically look at PI3K would be necessary.

Despite the above postulation, there has been previous work which promotes the notion that drug induced cardiotoxicity is associated with increases in Akt (Deres et al. 2005, Merten et al. 2006, Ichihara et al. 2007, Liu et al., 2008a, Gharanei et al., 2013). In particular, these studies have been in the context of doxorubicin associated myocardial injury, which have shown Akt phosphorylation to be up-regulated by up to 250% in comparison with control groups (Merten et al. 2006). As well as abrogation of Akt activation due to pre- or co-administration of a cardioprotective agent, such as CsA, with doxorubicin being associated with reduction in infarct size and improvements in haemodynamic function (Deres et al. 2005, Gharanei et al. 2014).

The promotion of ROS due to doxorubicin administration is postulated as promoting the activation of Akt and, in the context of this study it is proposed that the mechanistic similarities of ipratropium and doxorubicin, due to shared anti-muscarinic actions (Sasaki et al. 2010) are of relevance and that ipratropium may also be inducing Akt through a similar mechanism to that of doxorubicin. However, it has also been proposed that it is not the anticholinergic properties of doxorubicin which are responsible for ROS generation (Sasaki et al. 2010). In addition, there is a contemporary notion which suggests that muscarinic antagonists hold potential as anti-cancer therapies. Although the mechanisms remain unknown and are difficult to unravel due to the diverse phenotypes and genotypes of tumour cells, it is suggested that Akt and MAPK signalling as well as increases in ROS (as associated with I/R and doxorubicin induced myocardial injury) may play a role in the observed anti-cancer effect (Russo et al. 2014). Therefore it is currently difficult to ascertain whether there is a shared mechanism between ipratropium and doxorubicin induced cardio-toxicity.

It is also important to note that, following I/R, increases in Akt phosphorylation, despite being associated with increases in myocardial damage following doxorubicin treatment (Gharenai et al. 2014), may actually represent an endogenous protective mechanism to counteract against doxorubicin induced apoptosis (Taniyama and Walsh 2002, Ichihara et al. 2007). However this remains a matter of great debate as promotion of Akt activation has also been shown to protect against myocardial injury both in this context and, also, due to exogenous administration of Ach which has been shown to promote survival and salvage of myocardial tissue by Akt and Erk 1/2 activation (Taniyama and Walsh 2002, Wiklund et al. 2012). Erk1/2 may be a downstream kinase of Akt which is frequently associated with cardio-protection due to it's role in RISK signalling and, in the context of muscarinic signaling, has shown to elicit cardio-protective mechanisms (Hausenloy and Yellon 2004). It is proposed here that the observed increases of Erk1/2 following ipratropium administration are due to increases in Akt which, therefore lead to subsequent elevation of downstream Erk1/2 phosphorylation.

In this study, with the exception of a decrease following ipratropium administration at the 15 minute reperfusion time point, there were no observed differences in levels of JNK activation following ipratropium administration in the three month rats. Although JNK is not a direct downstream messenger of muscarinic signalling, it has a well defined role in the initiation of apoptosis as necrosis following I/R injury (Xu et al. 2014). It therefore appears as though downstream transcription of pro-apoptotic agents following JNK activation are not involved in ipratropium induced myocardial injury.

7.1.4 Differences in aged hearts

Within the aged studies (Chapter 6), it was identified that ipratropium induced significant increases in myocardial injury in the 12 and 18 month age groups in comparison with the 3 month old studies (Chapters 3, 4 and 5). However, it is also necessary to note that the control levels of injury due to I/R or oxidative stress alone, were also more severe. This implies that the aged myocardium is less resilient to injury, which supports previous work which shows protection from pre-conditioning and survival-signalling kinase pathways are lost following aging, leading to increased susceptibility to myocardial injury (van den Munckhof et al., 2013, Peart et al. 2014, Poulose and Raju, 2014). From a signalling perspective, it was also observed that there were significant increases in p-Akt and p-Erk1/2 following ipratropium administration in comparison with the respective untreated control of the same age group. However, it was observed that there was an age-related decrease in overall activation of these proteins which correlated with increasing age of the animals used. This supports previous work that shows that both Akt and Erk1/2 are activated at lower levels in the aged myocardium (Li et al. 2013, Peart et al. 2014) but, also, as the injury was more severe in these age groups in comparison with the 3 month studies, further supports that differences in Akt and Erk1/2 activation following ipratropium administration is an interesting consequence but not a necessary event for ipratropium induced injury to occur. Further to this, it has been demonstrated that GPCRs are under expressed and that downstream signalling is limited and less effective in the senescent myocardium (Willems et al. 2005). It is therefore likely that there is limitation on the protective mechanisms available following I/R injury and, in particular, an inability for the

myocardium to further salvage tissue following the extra insult of a cardio-toxic agent, such as ipratropium.

From the context of the experiments carried out using the model of oxidative stress, both of the aged groups (12 and 18 months) showed significant decreases in the time for depolarisation to occur in comparison with the 3 month groups. There was also no significant attenuation of injury (as observed in the three month groups) following administration of CsA or ACh. It is postulated here that this is due to age related differences in the ischaemic tolerance of the myocardium (Liu et al. 2002). There is increasing evidence to promote the “free radical theory of aging” (Boengler et al. 2009). This pertains to the mitochondria where, during aging, mitochondria are exposed to higher levels of ROS and, therefore enhanced oxidative stress, even under healthy conditions (Fernandez-Sanz et al 2015). Following I/R, these levels of oxidative stress are further enhanced and, as mitochondria in senescent cells are already swollen due to increased basal levels of ROS, this renders mitochondria less resilient to further oxidative stress, which is supported in this study as a significant reduction in time to depolarisation in the aged (12 and 18 month) studies, in comparison with the 3 month studies.

The inability of CsA or ACh to protect against a reduction in the time to depolarisation following sustained oxidative stress was expected as previous studies have also observed that there are age related differences in how CsA protects (Liu et al. 2011b). As ACh has been linked to the RISK pathway and mPTP, it is therefore likely that the inability for CsA to protect is due to a mechanism which links protective muscarinic signalling mechanisms to the mPTP and, from this respect, provides further evidence

that the pathways for CsA and ACh mediated cardio-protection are linked. However, in order to fully elucidate the events observed in the oxidative stress model, a potent uncoupler of oxidative stress, such as FCCP, would need to be employed as the present results cannot provide information as to whether ipratropium exacerbates depolarisation in this model or if, due to senescence, the myocytes are less resilient to oxidative stress and therefore there is no pharmacological intervention which can either protect against injury or exacerbate the observed injury.

In contrast to the work conducted in three month rats, in the aged rats, there was a significant increase in p-JNK levels following I/R as well as the groups where ipratropium was administered. JNK is a stress-induced kinase and is known to be endogenously activated at higher levels in senescent cells in comparison with comparative cells from younger age groups, irrespective of disease state (Shang et al. 2010). It is therefore postulated here that underlying, age related, increases in JNK activation may be partially responsible for the increase in I/R injury observed in the aged groups, however as there were no discernible differences in JNK activation following ipratropium administration (with the exception of the 15 minute reperfusion group in 3 month rats), it appears as though JNK does not play a role in ipratropium induced injury in the myocardium of either young (3 month) or aged (12 and 18 month) rat hearts following regional ischaemia and reperfusion.

It is also important to note that the pharmacokinetics of ipratropium have not been studied in elderly patients, despite this being the age group to which they are most commonly prescribed. Therefore, the aged study in this work is of particular

importance as, to our knowledge, it is the only study to have addressed the cardiovascular safety of ipratropium in a model of aging.

7.2 Study Limitations and future work

The aim of this study was to identify whether there was a basis for the clinical observations that pharmacological use of ipratropium promotes the occurrence and severity of adverse cardiovascular outcomes in patients with COPD. From a physiological perspective, this study has been successful as it has been identified that ipratropium is capable of exacerbating myocardial injury following simulated I/R and, in particular, that this effect appears to be restricted to reperfusion. This indicates that the presence of ipratropium during reperfusion interferes with signalling cascades and cellular events which occur during reperfusion (for example opening of the mPTP and proteins involved in the RISK pathway). However, within the scope of this study it has not been possible to fully elucidate the exact mechanism by which ipratropium exerts the observed cardio-toxic effects.

The primary limitation of this study is that as ipratropium is a non-selective antagonist, it has not been possible to identify whether there is an action on a specific subtype of muscarinic receptor. It has been shown that the myocardium contains M_1, M_2 and M_3 subtypes (Abrams et al. 2006). The use of specific muscarinic antagonists such as Telenzepine dihydrochloride (M_1), AF-DX 116 (M_2) and DAU 5884 hydrochloride (M_3) could assist in ascertaining whether there is a specific nature to the ipratropium induced injury. Although in previous work it has been speculated that it is likely due to the action on M_3 receptors as M_3 receptors appear responsible for the anti-apoptotic

properties of muscarinic activation (Pan et al. 2012, Wang et al. 2012, Hang et al. 2013), preliminary studies using the selective M_1 and M_3 antagonist, tiotropium bromide, did not indicate an exacerbation of injury (data not shown in this thesis). Although, this is in contrast to previously published clinical data which implies that tiotropium elicits higher levels of myocardial damage than ipratropium. However, the available data from the present *in vitro* study implies that it may be limitation of protective downstream signalling from the M_2 receptors which is responsible for the observed ipratropium induced injury. However, this remains unanswered and so further studies to classify which receptors are involved should be carried out.

Despite the ability of ACh to attenuate the ipratropium induced myocardial injury, there is insufficient evidence in this work to claim that the action of ipratropium is definitively due to a specific action at muscarinic receptors. In order to address this issue, further work with the use of acetylcholinesterase (AChE) would need to be carried out. As through this study endogenous levels of ACh were identified, it is currently postulated that ipratropium (as a competitive antagonist for acetylcholine at muscarinic receptors) essentially out-competes the endogenous ACh and, as such limits the downstream muscarinic signalling, thereby causing injury. The use of AChE would remove any ACh present and therefore, if administered in conjunction with ipratropium, it would be possible to ascertain whether ipratropium was still able to elicit injury. If this were the case, it would imply that ipratropium induced myocardial injury is as a result of off-target toxicity rather than a specific action at the muscarinic receptors.

In order to more fully identify the cellular events which occur, it would also be useful to carry out studies where connexin-43 (Cx-43) as well as the NCX are explored in the context of I/R and ipratropium administration. This is because it has been shown that ischaemia impairs the association of Cx-43 and muscarinic receptors which limits protective signalling and causes a cascade of injury due to contraction band necrosis (CBN) (Shintani-Ishida, Unuma and Yoshida 2009). Acetylcholine has shown a role in preserving these interactions (Yue et al. 2006) and, therefore, it may be that ipratropium limits this and promotes CBN. This would support the current study as myocyte loss due to ipratropium administration was identified as a consequence of both apoptosis and necrosis. Identification of involvement of the NCX would also help to clarify as within the cardiovascular system, it has been shown the M_3 activation by ACh decreases expression of the NCX-1 antiporter, which assists in the reduction of Ca^{2+} overload during reperfusion (Wang et al. 2012). As Ca^{2+} overload is a determining event in the opening of the mPTP, it is therefore possible that interaction of ipratropium at the M_3 receptors may conversely limit this reduction in cytosolic Ca^{2+} due to prevention of downstream down-regulation of NCX-1. This could potentially provide novel information which may help identify the role of the mPTP in ipratropium induced myocardial injury.

The final limitation of this study is that although clinically relevant models were used throughout, it was not possible to use an *in vivo* model and, in particular an *in vivo* model of COPD. Systemically, the interactions of ipratropium with other organs also need to be explored and, in particular, patients with any form of renal complications are excluded from clinical trials prior to drug release onto the market. Elderly patients

are also excluded, which, given the average age for a COPD diagnosis is 67 years (Goff et al. 2007) and that cellular signalling in the senescent myocardium is altered from that of young patients, implies that the effect of ipratropium induced myocardial injury has not been fully identified in the primary age groups to which this drug is prescribed.

7.3 Overall Conclusions

To conclude, the data presented here have identified for the first time that ipratropium is capable of eliciting myocardial injury following regional ischaemia and reperfusion. It is postulated here that it is the effect of ipratropium on signalling mechanisms which occur during reperfusion which are responsible for the observed injury. The observation that ACh is able to partially abrogate ipratropium induced injury implies that the effect is due to disruption in downstream, protective, cholinergic pathways which have been previously shown to protect the myocardium following an ischaemic insult (Resende 2007). In addition to this, CsA was also able to prevent some of the ipratropium induced injury which indicates that, at least in part, ipratropium induced myocyte loss is due to necrosis and apoptosis through a mechanism which involved the mPTP. The involvement of the pro-survival kinases Akt and Erk1/2 needs to be further characterised, however in this study, the increases observed following ipratropium treatment are attributed to an endogenous survival mechanism, activated to oppose the ipratropium induced injury, however, especially in the case of Akt, this may serve to exacerbate injury as constitutive over-action of Akt is detrimental to cellular survival. Despite these observations, the use of wortmannin entirely blocked the activation of Akt following ipratropium administration; however

this did not abrogate the observed increase in infarct development. Therefore it is postulated that although Akt, and subsequent downstream Erk1/2, activation may be a consequence of ipratropium administration, these proteins alone are not sufficient for injury to occur and ipratropium induced myocardial injury is not dependent on this signalling cascade. Interestingly, there were few observed differences in JNK phosphorylation in the 3 month rats, however there were significant increases in all groups in the aged studies. This is attributed to age-related differences in stress induced proteins (such as JNK) which may contribute to an increase in myocardial injury following ischaemia/reperfusion, even in the absence of ipratropium. The aged study provided further, novel information, which indicates that, in the elderly population, exacerbation of myocardial injury due to ipratropium is more severe. From a clinical perspective, it is necessary for practitioners to prescribe ipratropium, and other anti-cholinergic agents, with caution in patients who suffer from IHD or may be at risk of cardiovascular complications, especially given that in pre-clinical trials the elderly population are excluded.

According to Boehringer-Ingelheim, the manufacturer of ipratropium nasal spray (marketed as Atrovent®), the systemic bioavailability of ipratropium following oral administration is limited as ipratropium is unable to readily absorb across mucosal membranes. As such, following administration it would be postulated that concentrations of ipratropium in the blood would be zero, however this is not the case and plasma values which range between 5×10^{-8} – 1×10^{-7} M have been reported following a single, inhaled dose of Atrovent® (Singh et al. 2008). It is likely that this is due to around 90% of inhaled drugs being swallowed due to improper inhaler use. To

compound this issue, inhalers are also one of the most frequently abused medications with patients frequently deviating from their dose and administering the medication more often than advised. It is therefore likely that our observations from this work are clinically relevant with respect to both the concentrations used and the exacerbation of injury observed.

In the future, it is of paramount importance that the effect of muscarinic antagonists are fully characterised in disease models, particularly those of MI and COPD, as well as in healthy individuals prior to their release onto the market. In particular, clinically relevant models of elderly patients should be fully explored as differences in cellular signalling in senescent cells appear to play a major role in the ipratropium mediated exacerbation of myocardial I/R injury observed in this work.

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